A Dominant Suppressor Screen to Identify New Components in the TNF/Eiger-JNK-Pathway of *Drosophila melanogaster*

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Meinen Eltern Heidi und Bernhard Geuking gewidmet.

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Abbreviations

AP-1	activator protein 1
Bsk	Basket
CRD	cystein-rich domain
DD	death domain
Egr	Eiger
EMS	ethyl methanesulfonate
FADD	Fas-associated death domain protein
GCK	germinal center kinase
Hep	Hemipterous
IκB	inhibitor of kappa B
IKK	inhibitor of kappa B kinase
IL	interleukin
JNK(K)(K)	c-Jun-N-terminal kinase (kinase) (kinase)
LPS	lipopolysaccharide
MAPK(K)(K)(K)	mitogen-activated protein kinase (kinase) (kinase) (kinase)
MEKK	mitogen-activated protein kinase kinase kianse
Mkk	mitogen-activated protein kinase kinase
Msn	Misshapen
MyD88	myeloid differentiation primary response gene (88)
NFκB	nuclear factor kappa B
NIK	NFκB-inducing kinase
RIP	receptor-interacting protein
TAB2/3	TAK1 binding protein 2/3
TAK1	TGFβ activated kinase 1
TGFβ	transforming growth factor beta
THD	TNF homology domain
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRADD	TNF receptor-associated death domain protein
TRAF	TNF receptor-associated factor
Wgn	Wengen

1 Zusammenfassung

Cytokine, welche zur Superfamilie der Tumor Nekrosis Faktoren (TNF) gehören, sind im Menschen in zahlreichen physiologischen und pathologischen Prozessen involviert. Daher sind die TNF Signalmechansimen und die dadurch ausgelösten zellulären Antworten von hohem Interesse für die Forschung. Mehr als 30 Jahre intensiver Forschung haben ein Netzwerk von Signalwegen und zellulären Antworten hervorgebracht, welche durch TNF Liganden induziert werden. Um diesen komplexen Signalweg und seine Mitwirkung bei der Entstehung von TNF assozierten Krankheiten besser zu verstehen, ist es äusserst wichtig, alle Signal-komponenten und Regulatoren dieses Signalweges zu identifizieren. In Säugern ist die Erforschung von TNF-Signalmechansimen durch das Vorhandensein von mehreren Liganden und Rezeptoren und mindestens drei intrazellulär induzierten Signalwegen erschwert. Bis anhin wurden neue Komponenten vor allem mittels biochemischen Methoden oder Homologien zu bereits bekannten Komponenten identifiziert. Im Genom von *Drosophila melanogaster* ist nur ein einziges TNF Homolog (Eiger) codiert, was es erlaubt, nun auch genetische Methoden zur Entdeckung neuer Komponenten anzuwenden.

In Säugern werden durch TNF vor allem der NFκB (nuclear factor kappa B)-, JNK (c-Jun-N-terminal kinase)- und Caspase-8-Signalweg aktiviert, was zu verschiedensten Antworten wie Proliferation, Differenzierung, Überleben und Apoptose führen kann. Überexpression von Eiger im Facettenauge von *Drosophila melanogaster* löst lokale JNK-induzierte Apoptose aus, was zu einem dramatischen Verlust von Augengewebe führt. Dieser “Kleine-Augen-Phänotyp” war die Basis für die Entwicklung eines Testsystems zur Isolation dominanter Suppressoren dieser Aktivität. Zu diesem Zweck wurde ein Mutagenese-Screen durchgeführt, wobei über 100 Mutationen isoliert wurden, welche dominant den Eiger-JNK-Signalweg schwächen. Wie erwartet betreffen einige dieser Mutationen bereits bekannte Komponenten. Es wurden aber auch Mutationen in den Genen *dTAB2*, *Mkk4* und *lilliputian* gefunden, deren Genprodukte noch nicht mit dem Eiger-Signalweg assoziiert waren.

Wir konnten zeigen, dass dTAB2 eine Adaptorfunktion hat, welche die Rezeptorkomplex-Komponente dTRAF1 mit einer der aktiven Schlüsselkomponeneten dTAK1 verbindet. Dies demonstriert auch, dass die Funktion von TAB Proteinen von der Fliege bis zum Menschen konserviert ist. Unsere Experimente haben weiter gezeigt, dass, im Gegensatz zu Säugern, in *Drosophila* beide JNK aktivierenden Kinasen, Hemipterous (dMkk7) und Mkk4, für die TNF-induzierte Aktivierung von JNK benötigt werden. Ob im Menschen ein dem Protein Lilliputian ähnlicher Transkriptionsfaktor ebenfalls in Kooperation mit AP-1 das TNF Signal transduziert, wie das unsere Experimente in *Drosophila* andeuten, bleibt zu erforschen.

Das Klonieren weiterer Suppressoren und die Charakterisierung deren Genprodukte ist ein wichtiger Schritt in der Entschlüsselung dieses evolutionär konservierten Signalweges, was möglicherweise auch zur Entwicklung neuer Medikamente für die Behandlung von Auto-immunkrankheiten, Krebs und Diabetes führen kann.

2 Summary

Cytokines of the tumor necrosis factor (TNF) superfamily are involved in a vast number of physiological and pathological processes in humans. The signaling mechanisms and cellular outputs induced by TNF ligands are therefore of high interest, which is reflected by the immense amount of research performed in this area. Over 30 years of intensive studies has revealed a network of signaling pathways and cellular responses that are induced by TNF. To understand this complex signaling pathway, and its contribution to the development of diseases where TNF signaling is deregulated, it is important to identify all the regulators and transduction components. The study of TNF signaling mechanisms in mammalian systems is complicated by the existence of numerous ligands and receptors and at least three different intracellular signaling pathways. So far new components were mainly isolated by biochemical or homology search approaches. The identification of a single TNF family member, Eiger, in the genome of *Drosophila melanogaster*, offers the possibility to apply genetic approaches for pursuing this goal.

In mammals TNF mainly induces the NFκB (nuclear factor kappa B), JNK (c-Jun-N-terminal kinase) and caspase-8 pathways, leading to responses as diverse as proliferation, differentiation, survival and apoptosis. When overexpressed in the compound eye of *Drosophila melanogaster* Eiger induces JNK-mediated apoptosis, resulting in a dramatic loss of eye tissue. We used this “small eye phenotype” as the basis for an assay for dominant suppressors of this activity. In order to isolate rate-limiting components involved in Eiger-induced apoptosis a dominant mutagenesis screen was performed. Over 100 mutations that dominantly weaken the Eiger-JNK pathway were isolated. As expected, some of these mutations affected already known components of the Eiger pathway. Importantly we also isolated mutations in the genes *dTAB2*, *Mkk4* and *lilliputian*, encoding for proteins that had not yet been implicated in the Eiger pathway of *Drosophila*.

dTAB2 was shown to act as an adaptor molecule linking the receptor complex component dTRAF1 to one of the key signaling components dTAK1, demonstrating a conserved role for TAB proteins from flies to humans. Our experiments further suggest that in Drosophila both JNK activating kinases, Hemipterous (dMkk7) and Mkk4, are required for TNF-induced JNK activation, which is in contrast to the situation in mammals. Whether a human Lilliputian-like transcription factor also contributes to TNF-induced, AP-1-mediated transcription, as our results in *Drosophila* indicated, remains to be elucidated.

The mapping of the other suppressor mutations and the characterization of their corresponding gene products may uncover further important aspects of this evolutionarily ancient signaling pathway and possibly reveal new drug targets for the treatment of diseases like cancer, autoimmune disorders and diabetes.

3 Introduction

The existence of a cytokine with a tumor necrotizing potential, that is induced by LPS and mediates regression of tumors, was already described more than three decades ago (Carswell et al., 1975; Williams and Granger, 1968). However, only in 1984/1985 the first two members of the tumor necrosis factor (TNF) superfamily, TNF-α and TNF-β, have been purified and cloned (Aggarwal et al., 1985a; Aggarwal et al., 1985b; Aggarwal et al., 1984; Gray et al., 1984; Pennica et al., 1984). They represent the two founding members of the TNF superfamily, currently consisting of 19 ligands. Several years later the first receptors belonging to the tumor necrosis factor receptor (TNFR) superfamily, now consisting of 29 members, were identified. TNF ligands are type II transmembrane proteins (intracellular N-terminus and extracellular C-terminus) containing a characteristic “TNF homology domain” (THD) in the C-terminal part of the protein. The THD is required for receptor binding and trimerization of the ligand. In some cases cleavage of membrane-bound ligands by metalloproteases is required in order to generate active soluble ligands. TNFRs are type I or type III (lack of a signal peptide) transmembrane proteins, which have an intracellular C- and an extracellular N-terminus. These receptors carry between one and six characteristic “cysteine-rich domains” (CRD) in their extracellular part. CRDs are involved in ligand binding. The TNFR superfamily is subdivided into two subgroups: The so-called death-receptors, that carry an intracellular death-domain (DD) and a second group lacking the DD. Death-receptors bind Jun-N-terminal kinase (JNK)-inducing TRAF proteins through the DD-containing adaptor molecule TRADD, which binds via homophilic interaction to the receptor. The Caspase-8-recruiting, DD-containing adaptor molecule FADD can either directly (e.g. FAS) or indirectly via TRADD (e.g. TNFR-1) bind to a death-receptor. Some of the TNFRs lacking a DD bind directly to TRAFs (e.g. TNFR-2), but all of them fail to bind to TRADD or FADD and therefore fail to induce Caspase-8-dependent apoptosis. Both, ligands and receptors, have to form a homotrimer to become active signaling components (Aggarwal, 2003; Wajant et al., 2003).

3.1 Biological Functions of TNF

TNF ligands play prominent roles in the regulation of physiological processes, such as the immune system, inflammation, haematopoiesis and the development of hair follicles, sweat glands and neuronal networks (Locksley et al., 2001). However, deregulation of TNF signaling is associated with pathological conditions, including autoimmune disorders, cancer and diabetes (Aggarwal, 2003; Chen and Goeddel, 2002). A bigger picture of biological functions of TNF is depicted in Figure 1. For some of these diseases TNF ligands and receptors are being evaluated as drug targets for therapy. TNF-α inhibitors (Etanercept or Infliximab) for example have been proven to be very effective in treatment of rheumatoid arthritis (Luong et al., 2000), but for therapy of multiple sclerosis it turned out to have deleterious effects (Kassiotis and Kollias, 2001). The cellular outputs of the TNF signal are as widespread as the conditions mentioned above. Activation of TNFRs induces several intracellular signaling pathways that can lead to apoptosis, survival, proliferation and differentiation. The high number of members belonging to the TNF- and TNFR-superfmailies and the pleiotropic functions of TNF clearly highlight the complexity of TNF biology in mammals. This complexity is further enhanced by the crosstalk occurring between the different intracellular signaling pathways that are induced by TNFRs.

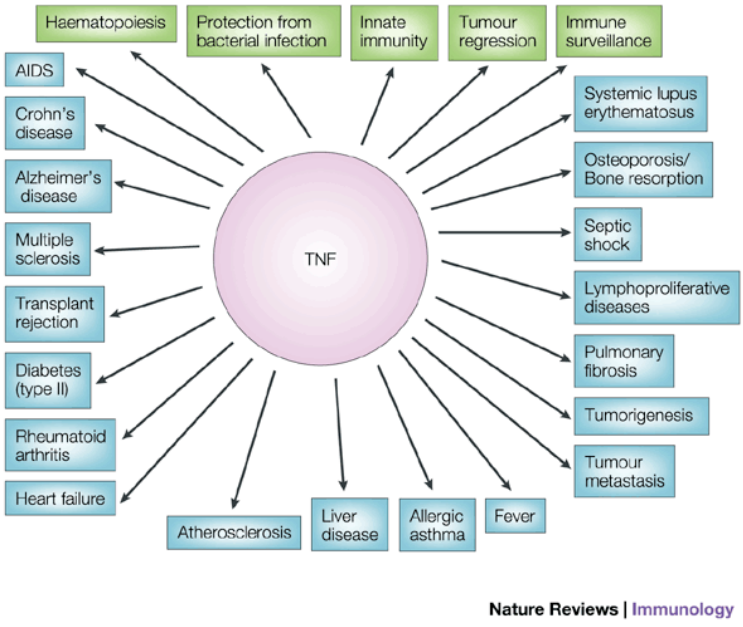


Figure 1 Physiological (green) and pathological (blue) processes influenced by TNF signaling are indicated (Aggarwal, 2003).

3.2 Signaling Pathways Induced by TNF

Primarily three intracellular pathways are activated upon binding of TNF ligands to their receptors. An overview of the components involved in the activation of these pathways given in this section is by far not complete, but reflects the present view of the “canonical” TNF pathway (Figure 2). Not all TNFRs induce all three pathways, but only one or two of them. In addition there are also other “non-canonical” pathways reported (p38, PKB-Akt, Ras-Raf) that are induced by a subset of TNF ligands. Activation of the transcription factors NFκB and AP-1 as well as induction of the initiator caspase-8 are the three canonical pathways briefly described in the following paragraph (Wajant et al., 2003).

(1) NFκB activation: Recruitment of RIP into the receptor complex is a crucial step in the induction of NFκB. RIP is involved in the activation of IKK, an inhibitor of IκB. In non-stimulated cells IκB masks the nuclear localization signal (NLS) present in NFκB. Phosphorylation of IκB by IKK marks it for proteasomal degradation, thereby allowing NFκB to enter the nucleus and activate transcription of target genes. Actvation of NFκB plays a role in the immune responses and is a survival signal for the cell. This TNF-induced survival signal is the reason why a lot of cell-types actually do not undergo apoptosis upon TNF stimulation (Wajant et al., 2003).

(2) AP-1 activation: TRAF2 is required to promote the induction of the JNK pathway upon TNF stimulation. How exactly TRAF2 mediates TNF-induced activation of JNK is not very well understood. Germinal center kinases (GCK) have been shown to bind to TRAF2 and to be able to activate different JNKKKs. Activation of the JNK signaling cascade finally leads to the phosphorylation of the transcription factor AP-1 consisting of c-Jun and c-Fos. While TNF-induced JNK signaling is generally believed to be important in the regulation of immune responses (Wallach et al., 1999), its role in apoptosis is controversial. JNK signaling is reported to have pro- as well as anti-apoptotic effects (Deng et al., 2003; Liu et al., 1996; Natoli et al., 1997).

(3) Caspase-8 activation: Recruitment of the initiator caspase-8 by FADD is the initial step for activation of the effector caspase-3. This way of induction of programmed cell death, apoptosis, is also known as the extrinsic apoptotic pathway. Another mechansim is the intrinsic apoptotic pathway, which is activated by intrinsic signals, such as DNA damage. The apoptosome, consisting of caspase-9 and Apaf-1, is the key player of the intrinsic apoptotic pathway. Mitochondria play an important regulatory role in the intrinsic pathway (Hengartner, 2000).

A pathway mechanistically very similar to the TNF pathway is the TLR (Toll-like receptor) pathway. It also plays a prominent role in the immune system. In the TLR pathway TAK1 is the major inducer of NFκB as well as JNK (Kawai and Akira, 2006). The importance of NIK (NFκB-inducing kinase) in NFκB activation is still not fully understood. In general, the main difference between the TLR and TNF pathway is that, due to the lack of a DD, TLRs fail to directly recruit FADD to induce caspase-8-dependent apoptosis (Figure 2). However, exceptions are known bringing the TNF and TLR receptors mechanistically even closer together indicating that they share very similar signaling modules that possibly have been adopted independently during evolution. TLR-2 for example has been shown to induce caspase-8-dependent apoptosis by homophilic DD-mediated binding of FADD to MyD88 (Aliprantis et al., 2000).

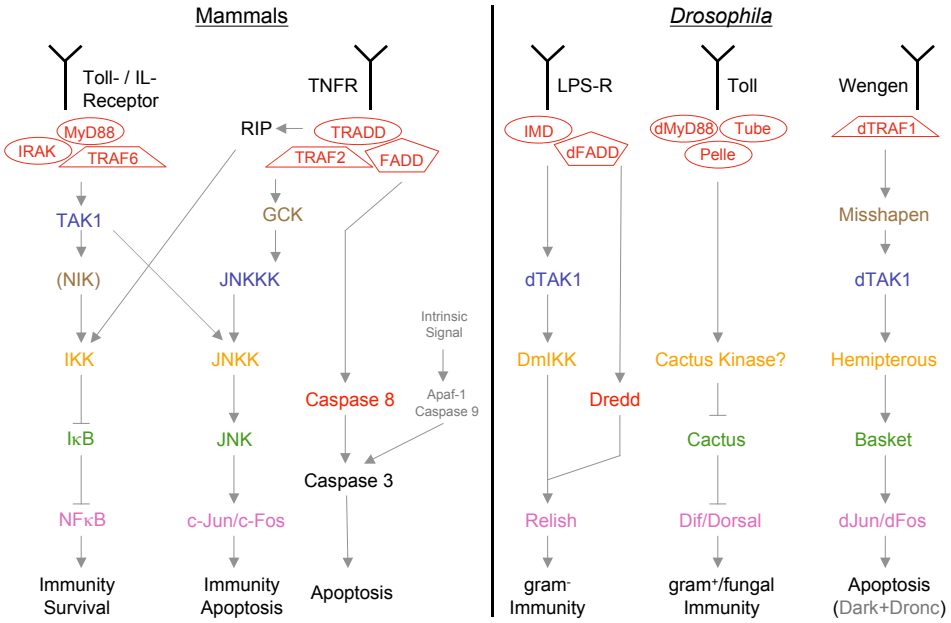


Figure 2 TNF- and TLR-signaling in mammals and *Drosophila*.

3.3 TNF/Eiger Signaling in *Drosophila*

In the genome of *Drosophila melanogaster* a single gene is predicted that encodes a protein with homology to mammalian TNFs. Eiger, the 415aa product of this gene, contains a N-terminal transmembrane domain and a C-terminal THD, both characteristic for TNF ligands. Overexpression of *eiger (egr)* in the *Drosophila* compound eye leads to a dramatic ablation of eye tissue (Figure 3) (Moreno et al., 2002b). Interestingly, in flies homozygous mutant for Dredd (*Drosophila* caspase-8) or dFADD (not shown) Eiger is still able to induce a small eye phenotype (Moreno et al., 2002b), indicating that in *Drosophila* the FADD-caspase-8 module is not required for Eiger-induced loss of eye tissue. But it is important to note, that Dredd and dFADD are involved in a Drosophila Toll-like pathway, called IMD (Leulier et al., 2000; Naitza

et al., 2002). The IMD pathway leads to the activation of Rel, one of the *Drosophila* homologs of NFκB, and mediates innate immunity against gram-negative bacterial infection (Hultmark, 2003). This is an exciting observation suggesting that early in evolution the FADD-caspase-8 branch was used by TLRs and only later was adopted by the TNF system. The role of mammalian FADD and caspase-8 in NFκB activation has not been properly elucidated. The situation again seems to be more complex than in *Drosophila*, indicated by some reports claiming FADD-caspase-8 to be an inducer of NFκB activation (Chaudhary et al., 2000; Hu et al., 2000; Rathore et al., 2004) while others suggest an inhibitory role for FADD-caspase-8 in NFκB activation (Bannerman et al., 2002; Duckett, 2002).

puckered (puc), a target gene of the JNK pathway in *Drosophila*, encodes a dual-specificity phosphatase that acts as a feed-back inhibitor of the pathway by directly inhibiting Basket (Bsk, *Drosophila* JNK) activity (Martin-Blanco et al., 1998). Co-expression of Puc together with Eiger completely suppressed the small eye phenotype strongly suggesting that Eiger only acts through Bsk. Removing one or two copies of components of the Drosophila JNK pathway (*msn* (Igaki et al., 2002; Moreno et al., 2002b), *dTAK1* (Geuking et al., 2005), *hep* (Igaki et al., 2002), *bsk* (Igaki et al., 2002; Moreno et al., 2002b), and *djun* (Appendix)) also suppressed the small eye phenotype confirming its role in Eiger-induced eye tissue ablation. In addition a deficiency that deletes *dTRAF1* (encoding the homolog of TRAF2) also dominantly suppressed the small eye phenotype (Moreno et al., 2002b).

The main transducer of apoptosis in flies is the *Drosophila* counterpart of the mammalian apoptosome consisting of Dark (*Drosophila* Apaf-1) and Dronc (*Drosophila* caspase-9). In non-dying cells *Drosophila* caspases are bound by “inhibitor of apoptosis proteins” such as DIAP1. So far there are at least five pro-apoptotic genes known in Drosophila, of which all induce apoptosis by releasing Dronc from its inhibition by DIAP1 (Meier et al., 2000). These genes are *head involution defective (hid)* (Grether et al., 1995)*, reaper (rpr)* (White et al., 1996)*, grim* (Chen et al., 1996)*, sickle* (Christich et al., 2002; Srinivasula et al., 2002; Wing et al., 2002) and *jafrac2* (Tenev et al., 2002). All of them except *jafrac2* are encoded at the same locus in the *Drosophila* genome (75C). The discovery of a deficiency (*Df(3L)H99*) deleting three of them, *hid, rpr* and *grim,* was the starting point for the research field of developmental apoptosis in *Drosophila* (White et al., 1994). In flies possibly all apoptosis-inducing events, such as DNA damage or developmental apoptosis, lead to the transcriptional upregulation of one or more of these pro-apoptotic genes (Meier et al., 2000).

Several experiments clearly indicated that overexpression of Eiger in the *Drosophila* eye indeed induces Dronc-dependent apoptosis. Co-expression of DIAP1 or a dominant negative form of Dronc (CARD) suppresses the small eye phenotype. In addition, flies heterozygous for *dark* or *Df(3L)H99* also display a suppressed small eye phenotype. The involvement of pro-apoptotic genes was further confirmed by detection of transcriptional upregulation of *hid* upon Eiger-induced JNK signaling (Moreno et al., 2002b).

In summary, Eiger activates the JNK pathway, which in turn leads to transcriptional upregulation of at least one of the pro-apoptotic genes (*hid*). Subsequently Hid releases Dronc from its inhibition by DIAP1 leading to the initiation of programmed cell death (Figure 3).

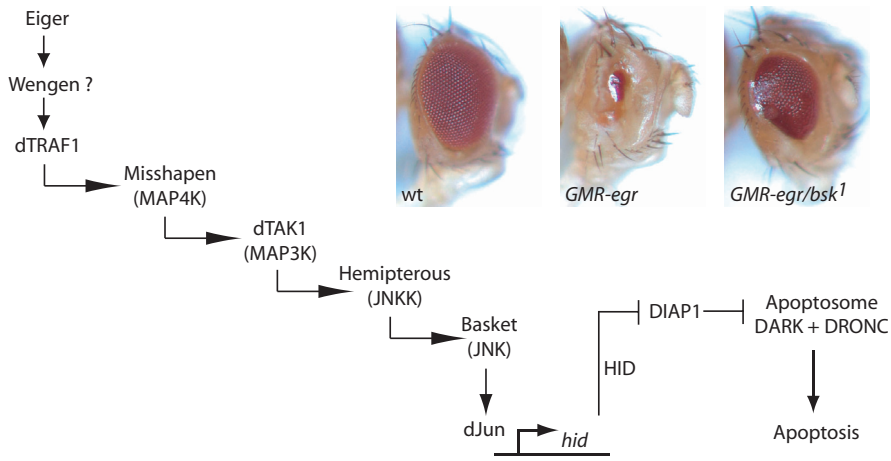


Figure 3 Eiger overexpression in the *Drosophila* compound eye leads to JNK-mediated transcriptional upregulation of *hid* and subsequent induction of apoptosis.

In mammals one way for NFκB to exert its anti-apoptotic/survival function is by inducing the transcription of genes whose products were shown to directly inhibit the JNK pathway (De Smaele et al., 2001; Tang et al., 2001b), assigning a pro-apoptotic rather than an anti-apoptotic role to the JNK pathway. When NFκB is blocked TNF's potential to induce apoptosis increases dramatically (Wajant et al., 2003). One such a NFκB target gene is *gadd45β.* Gadd45β directly inhibits JNK signaling by targeting Mkk7 (Papa et al., 2004a; Papa et al., 2004b). However, when the closest structural *Drosophila* homolog (CG11086) was co-expressed with Eiger, no inhibiting effect was observed (not shown). Whether the Drosophila homologs of NFκB (Relish, Dorsal, Dif) have an inhibitory effect on Eiger signaling remains to be elucidated. Relish indeed was reported to have an inhibitory effect on dTAK1 in LPS-induced JNK activation (Park et al., 2004). The anti-apoptotic role of NFκB is thought to depend on its simultaneous activation together with induction of apoptosis by TNF. Since no activation of NFκB by Eiger was observed so far (Igaki et al., 2002), a physiological crosstalk between NFκB and JNK in Eiger signaling is rather unlikely.

Since the *Drosophila* genome encodes only a single homolog of TNF, it was obvious to look for a homolog of a TNFR as well. Indeed, such a search was successful and lead to the identification of a single Drosophila TNFR homolog called Wengen (Wgn) (Kanda et al., 2002; Kauppila et al., 2003; Röthlisberger, 2002). Wengen is a 343aa type III transmembrane protein with a characteristic N-terminal CRD. However, unlike mammalian TNFRs, Wengen contains neither a DD nor a consensus TRAF binding motif. Co-expression of a UAS-RNAi-hairpin construct of *wgn* as well as heterozygousity for a deficiency uncovering the *wgn* locus (*Df(1)E128*) was reported to suppress the Eiger-induced small eye phenotype (Kanda et al., 2002). Also RNAi experiments in cell culture indicated a ligand-receptor relation for Eiger and Wengen (Kauppila et al., 2003). While Kanda et al. (2002) claimed to observe a physical interaction between Eiger and Wengen, Kauppila et al. (2003) were not able to reveal such an interaction. However, the *in vivo* experiments performed by Kanda et al. (2002) could not be reproduced. Both, co-expression of a *UAS-wgn-hairpin* transgene as well as heterozygousity for *Df(1)E128,* did not suppress the small eye phenotype in our hands (not shown). More importantly, a targeted knock-out allele of *wgn* did, even in a homozygous state, not suppress the Eiger-induced small eye phenotype (Röthlisberger, 2002). Aside from the conclusion that Wengen is not the receptor of Eiger, it is possible that there is redundancy at the level of the receptor. Whether an unknown type of receptor is also involved in the primordial TNF signaling cascade would be an interesting finding that remains to be investigated.

So far new components in the TNF signaling network were mainly identified by biochemistry or on the basis of homology searches. The existence of a single *Drosophila* TNF homolog offers the possibility to apply also genetic approaches to study its signaling mechanisms. In this thesis a genetic screen with the goal to identify novel components in the Eiger pathway is described. The identification and characterization of new components, such as novel receptors, may shed light on, as yet, unknown mechanisms of TNF signal transduction as well as on new therapeutic opportunities for the treatment of diseases where TNF signaling is deregulated.

4 Results

4.1 The EMS Screen

Based on the fact that removing one copy of known components of the *Drosophila* JNK pathway is sufficient to suppress the Eiger-induced small eye phenotype (Figure 3) (Igaki et al., 2002; Moreno et al., 2002b), we designed a screen for dominant suppressors of this phenotype (Geuking et al., 2005). By crossing mutagenized chromosomes into the “small eye” genetic background (*GMR-Gal4 UAS-egr*, in this thesis called *GMR-egr*), the progeny of such a cross can easily be screened for dominant suppressors. In a pilot screen, where *yw* males were mutagenized and mated with virgins of the following genotype, *yw*; *GMR-egr* / *CyO* ; *y+* / *y+* , approximately 5’000 chromosomes were screened and 21 suppressors were identified. Importantly the pilot screen confirmed that dominant suppressors can be identified with this setup.

However, using virgins of the *GMR-egr* stock for these crosses also leads to various technical problems. Since the *GMR-egr* stock is very weak in viability, it is hard to get enough virgins to run the screen efficiently. Flies homozygous for the *GMR-egr* chromosome are lethal and also heterozygous animals display reduced viability (i.e. not all of them hatch). This already reduces the efficiency of the screen dramatically. Furthermore only 50% of the screened F1 progeny carries the right genotype, the other 50% will carry the *CyO* balancer. It is therefore very time consuming to first sort out the flies with the correct genotype. The *y+* insertion on the third chromosome is used as a marker in the process of mapping the isolated suppressor mutations to a chromosome (Geuking et al., 2005). To improve the efficiency of the screen we subsequently mutagenized males carrying the *GMR-Gal4* insertion on the second chromosome (*yw*; *GMR-Gal4*; +). These males were then mated with virgins carrying a *UAS-egr* and a *y+* insertion on the second and third chromosome, respectively (*yw*; *UAS-egr*; *y+*). Both stocks display a high fitness/viability and with this setup 100% of the F1 progeny has the right genotype to be screened. It is important to mention that with this setup some of the suppressors that will be isolated will carry mutations in the *GMR-Gal4* transgene. But those can easily be identified based on two criteria (Geuking et al., 2005):

1) The suppressor mutation cannot be separated from the *GMR-Gal4* insertion by recombination.

2) The unrelated big eye phenotype induced by expression of *UAS-Inr* under control of *GMR-Gal4* (Brogiolo et al., 2001) is also reverted if the *GMR-Gal4* transgene is mutated.

Using the above described setup approximately 55'000 flies were screened and 117 suppressors were isolated in total (1 on X, 87 on II, 29 on III). This corresponds to 0.21% of the total number of screened flies and represents the normal yield of an EMS screen. That only one mutation on chromosome X was obtained is due to the genetics of the screen, which only allows one to isolate viable mutations on chromosome X, which eventually can even be lost again (Geuking et al., 2005).

In order to characterize the identified suppressor mutations, complementation analysis with known components of the pathway was conducted, namely, using *misshapen* (*msn*), *basket* (*bsk*), *djun* (*jra*) and *dfos* (*kayak*). All suppressor mutations that mapped to the second chromosome were tested for complementation with *bsk* and *djun*, which are also located on the second chromosome. Equally, all hits on the third chromosome were tested using *msn* and *dfos* alleles. Although mutations in *djun* and *dfos* do not dominantly suppress the Eiger-induced small eye phenotype (not shown), they were included in this analysis because of the possibility that strong or dominant negative alleles of *djun* or *dfos* could have been isolated in the screen. *djun* and *dfos* were shown to be involved in the Eiger pathway by other genetic experiments and RNAi in a cell-culture-based reporter assay (see Appendix and Chapter 4.7).

The only lethal complementation group we identified in this complementation analysis is a group of ten suppressor mutations that failed to complement the lethality of a *bsk* loss of function allele (bsk^1). Sequencing the *bsk* coding region of these mutants revealed molecular lesions in all of them (Geuking et al., 2005) confirming that our screening setup indeed works. At the same time this result revealed that we did not isolate mutations in *djun*, *dfos* or, surprisingly, *msn*. One explanation for not having obtained mutations in *msn* could be that the suppression brought about by dominant loss of *msn* is below the phenotypic threshold we screened for. Indeed, the suppression of the small eye phenotype observed by dominant loss of *msn* is rather weak (Igaki et al., 2002; Moreno et al., 2002b). Another explanation is that, e.g. depending on the chromatin structure, EMS may have different access to different chromosomal regions. *hep* and *dTAK1*, two further known components of the Eiger pathway, were excluded from this analysis because they are located on the X chromosome. *hep* null mutants are homozygous lethal, whereas *dTAK1* mutants are homozygous viable. In any case it is not possible to perform a lethality-based complementation analysis for genes on the X chromosome. Only one suppressor mutation located on chromosome X was isolated in the screen (*G14*). This suppressor behaved exactly as *dTAK1* mutants: it was homozygous viable and recessively completely suppressed the small eye phenotype. Genetic rescue experiments and sequencing of the *dTAK1* coding region in this suppressor confirmed it to be a novel *dTAK1* allele (Geuking et al., 2005).

Epistasis experiments with *GMR-hid* (*GMR-Gal4 EP-hid*) indicated that all suppressor mutations act genetically upstream of *hid* transcription: none of the mutations suppressed the small eye phenotype induced by *hid* expression in the eye (not shown). This phenotype was strongly suppressed by co-expression of a dominant negative version of Dronc (*UAS-CARD*) (Figure 4) proving that this phenotype is indeed dependent on the apoptotic machinery. A mutant of the initiator caspase *dronc* (*dronc*^{51}) (Chew et al., 2004), which acts downstream of *hid* in the pathway, does not dominantly suppress either *GMR-egr* or *GMR-hid* (not shown). Therefore it was not surprising that all suppressor mutations from the screen complemented the lethality of *dronc*^{51}, as it is consistent with the conclusion that all these mutations act genetically upstream of *hid* transcription. For epistasis experiments with a constitutive active form of Hep see the specific result sections for the genes identified in the screen (Chapter 4.5, 4.6, 4.7) and Appendix.

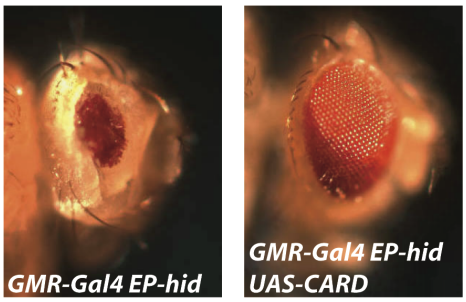


Figure 4 The small eye phenotype induced by expression of *EP-hid* under the control of *GMR-Gal4* is strongly suppressed by co-expression of a dominant negative form of Dronc (*UAS-CARD*).

4.2 The Deficiency Screen

In parallel to the EMS screen we screened the whole deficiency collection from Exelixis (Parks et al., 2004) and a subset of deficiencies created by the DrosDel project (Ryder et al., 2004) for dominant suppressors of the small eye phenotype. The Exelixis collection alone uncovers approximately 56% of the *Drosophila* genome. These deficiencies are in an isogenic background and their breakpoints are molecularly mapped, which is a major advantage compared to other deficiency collections available. The purpose of screening these deficiencies was to identify chromosomal regions important for Eiger signaling that possibly are weakly accessible by EMS. In addition the isolation of deficiencies that dominantly suppress the small eye phenotype will be very useful in order to map the EMS-induced suppressor mutations.

The testing of approximately 450 Exelixis and 140 DrosDel deficiencies revealed a total of six deficiencies with a medium to strong potential to suppress the small eye phenotype (Table 1 and Appendix). Two of the deficiencies, *Df(3L)ED224* and *Df(3L)ED225*, are overlapping and uncover the genomic region 75C of the well known pro-apoptotic genes *head involution defective* (*hid*), *reaper* (*rpr*), *grim* and *sickle* (*skl*) confirming their role in Eiger-induced apoptosis. *Df(3L)ED224* and *Df(3L)ED225* overlap in only six genes including *grim* and *rpr* but not *hid* and *skl* (Fugure 75C). *Df(3L)ED225*, which does not delete *hid*, indicates that Eiger-induced apoptosis is not only mediated by *hid*, but also by at least one of the three other pro-apoptotic genes. Another deficiency, *Df(3R)Exel6149*, is included in *Df(3R)ED5296* and in this case narrows the region of interest down to 27 genes. *Df(2R)Exel6069* and *Df(3L)Exel6107* delete only 20 and 25 genes, respectively. The suppression observed with *Df(2R)Exel6069* was confirmed with *Df(2R)BSC26* that uncovers the same region but in addition extends proximally and distally (Table 1). *Df(3L)Exel6107* failed to be confirmed with corresponding deficiencies (*Df(3L)ZN47* and *Df(3L)CH20*, discussed in Chapter 4.10). None of the deficiencies suppressed the small eye phenotypes observed with *GMR-hid* or *GMR-hep*^{CA} indicating that the responsible genes act in parallel or upstream of Hep (not shown). Testing all available loss of function alleles of the genes deleted in any of the three small Exelixis deficiencies for suppression of the small eye phenotype did not reveal the identity of the responsible genes. Furthermore, all mutations we obtained from the EMS screen also complemented the lethality of each of these three deficiencies. Therefore, either none of the EMS mutations affects genes disrupted in any of these three deficiencies or loss of function mutations of the affected genes are homozygous viable.

Deficiency	**Confirmed with**	**Comments**
Df(3L)ED224/Df(3L)ED225	each other	deletes locus 75C
Df(2R)Exel6069	*Df(2R)BSC26*	20 genes deleted
Df(3R)Exel6149/Df(3R)ED5296	each other	27 genes deleted
Df(3L)Exel6107	failed to be confirmed!	25 genes deleted

Table 1 Deficiencies isolated in the deficiency screen.

4.3 Genetic Rough Mapping

Since neither complementation analysis with identified deficiencies nor a partial complementation analysis within the pool of EMS mutations itself revealed any further lethal complementation group, we decided to first roughly map all the mutations relative to a genetic marker in order to subdivide them into different groups. Suppressors belonging to the same group can then be tested in a complementation analysis again. ~90% of the chromosomes isolated from our EMS screen are homozygous lethal. This is not necessarily due to the suppressor mutation itself, but possibly due to second lethal hits on the chromosome.

To map second chromosomal suppressor mutations we simply used the *GMR-Gal4* insertion as a genetic marker. By inverse PCR the *GMR-Gal4* driver was determined to be inserted at 57F6. Chromosomes carrying the *GMR-Gal4* insertion and a suppressor mutation were allowed to recombine with a wildtype chromosome in females. Such virgins were crossed to *UAS-egr* males. The number of *GMR-Gal4* progeny (marked by a “dark red” w^+ versus the weak “orange” w^+ of the *UAS-egr* insertion) with a suppressed eye phenotype in relation to the number of *GMR-Gal4* progeny with a small eye phenotype reflects the relative genetic distance between the suppressor mutation and the *GMR-Gal4* insertion. The closer a mutation is to the *GMR-Gal4* insertion the less likely it is that they will be separated by recombination (Figure 5a).

A similar analysis was performed for the few mutations that were obtained from the pilot screen, where wildtype chromosomes were mutagenized, The “wildtype” chromosome carrying a suppressor mutation was allowed to recombine with the *GMR-Gal4* chromosome. When again crossing such virgins back to *UAS-egr* males the progeny was analyzed in a reciprocal way than described above (Figure 5b).

It is important to notice that the distance values that are obtained with this method are only relative and not absolute values. It even turned out that only values obtained by the same crossing scheme can be compared with each other. This is probably due to the fact that the fitness of a *GMR-Gal4* chromosome that does not carry a suppressor mutation is much weaker when crossed back to a *UAS-egr* stock.

This analysis revealed three major groups of suppressor mutations on the second chromosome defined by their relative genetic distance (in % rgd, see Figure 5) to the *GMR-Gal4* insertion. (1) 0 rgd, (2) 2-8 rgd, (3): 28-36 rgd. Another class is represented by the new *bsk* alleles discussed earlier. These were subjected to this analysis as a control and mapped 25-33 rgd away from the *GMR-Gal4* insertion. Since the insertion is located on 2R and *bsk* on 2L, values higher than 25 rgd may already reflect uncoupled inheritance. As mentioned before, the values for the mutations obtained in the pilot screen differ from these, but they could also be used to determine whether a mutation is in close proximity to the locus where

the *GMR-Gal4* transgene is inserted or located on the opposite chromosome arm 2L. Values of 12-23 rgd and 69-124 rgd obtained by crossing scheme 5b belong to group 2 and 3, respectively (see below). The exact value obtained for each suppressor mutation is listed in the Appendix.

Group 1 reflects mutations in the *GMR-Gal4* transgene. All but five of them also reverted the unrelated *Inr* big eye phenotype and therefore fulfill the two criteria mentioned earlier (see Appendix). That the other five mutations affect a gene that just lies very close to the *GMR-Gal4* insertion is rather unlikely, because, in these special cases, a very high number of progeny was screened for a recombination event. One possible explanation for not suppressing the Inr-induced big eye phenotype is, that these mutations just weaken the *Gal4* expression to an extent that the *UAS-Inr* transgene is still sufficiently activated, but not the *UAS-egr* transgene.

Group 2 seemed to be located in proximity of the *GMR-Gal4* insertion. In theory, by comparing the chromosomal locations, these mutations could affect a gene deleted in *Df(2R)Exel6069*. Indeed, using overlapping deficiencies and sequencing candidate genes we identified mutations in *dTAB2* in 39 of these 42 stocks. For a detailed description see Chapter 4.5.

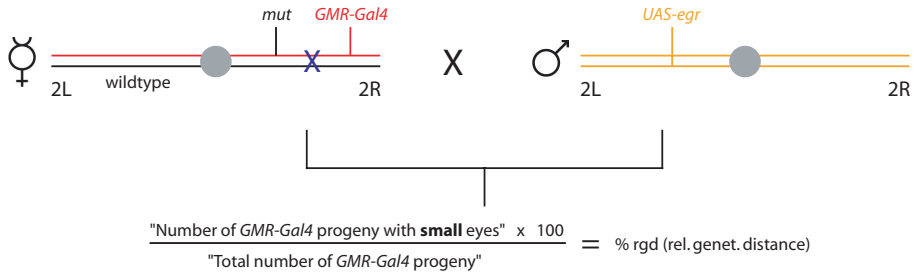
Group 3 reflects mutations located on 2L, which complement the lethality of *bsk* alleles. Complementation analysis with the five suppressor mutations of this class revealed that four of them fail to complement each other, and therefore represent a further lethal complementation group called *L1*. For detailed characterization of *L1* see Chapter 4.6.

To map the third chromosomal mutations in a similar approach, three RFP (red fluorescent protein)-marked insertions (J. Bischof, unpublished) were used as genetic markers. Two of them (*RFP15*@85E, *RFP4*@62B) were chosen due to proximity to *Df(3R)Exel6149* and *Df(3L)Exel6107* located at cytological position 85A2-5 and 64E5-F6, respectively. These deficiencies were identified as suppressors in the deficiency screen described earlier. The third one, *RFP5*, is inserted at position 92A (Figure 10). Again, mutated chromosomes were allowed to recombine with the RFP-marked chromosome in females. These virgins were crossed back to *GMR-egr*/*CyO* males. The number of non-*CyO* RFP progeny (sorted under the fluorescence binocular) with a suppressed eye phenotype in relation to the number of non-*CyO* RFP progeny with a small eye reflects the relative genetic distance to the RFP insertion (Figure 5c).

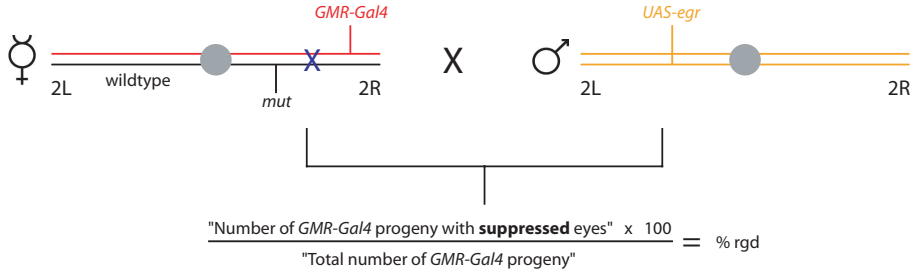
This analysis revealed that 23 of the 29 third chromosomal suppressor mutations are located very close to *Df(3R)Exel6149* (see chapter 4.7). The relative genetic distances to the RFP insertions obtained for all third chromosomal mutations are listed in the Appendix.

Rough mapping relative to genetic markers

a) Suppressor mutation located on *GMR-Gal4* chromosome (II):



b) Suppressor mutation located on wildtype chromosome (II):



c) Suppressor mutation located on chromosome (III):

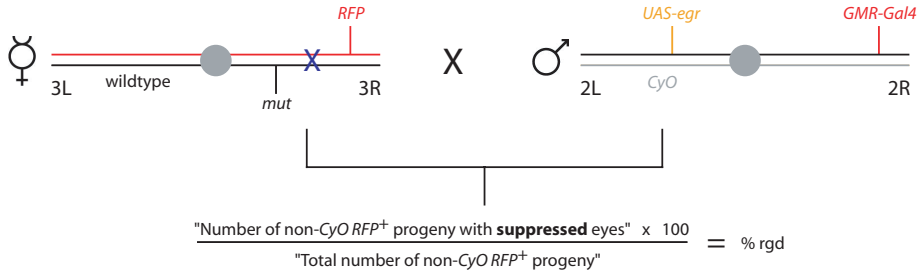


Figure 5 Crossing schemes for genetic rough mapping relative to a genetic marker.

4.4 Genetics of FLP Mapping

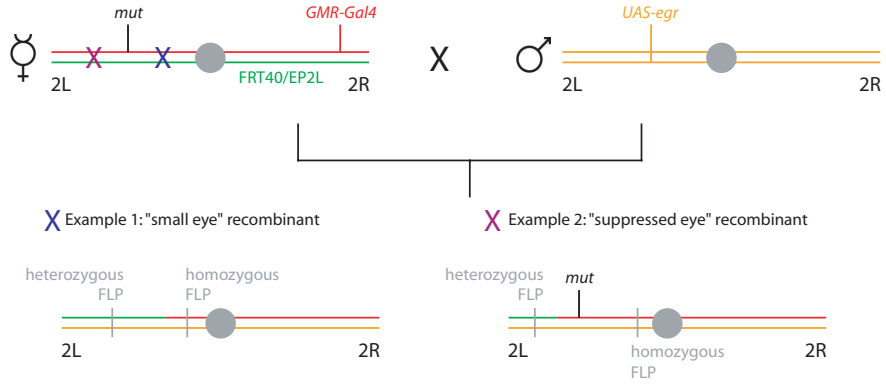
Second chromosomal EMS-induced suppressors that could not be mapped with the help of deficiencies identified in the deficiency screen were subjected to high resolution FLP (fragment length polymorphism) mapping. With this technique FLPs present between the mutated chromosome (*GMR-Gal4* or wildtype) and a reference chromosome (EP2L or FRT40) are used as genetic markers. For a detailed description of this method see Zipperlen et al. (2005).

In order to obtain suitable recombinants that can be analyzed with this technique the following genetic procedure was applied. The mutant chromosome was allowed to recombine with the reference chromosome in females. These virgins are crossed back either to *UAS-egr* or *GMR-egr* males, depending on whether the *GMR-Gal4* or the wildtype chromosome was mutagenized (Figure 6). The FLPs of single flies of the progeny displaying either a small eye or a suppressed phenotype were analyzed for hetero- or homozygousity. Due to the lack of a suitable additional marker, it was not possible to identify recombination events already before the analysis. But it turned out that a large number of the analyzed single flies indeed carried a recombination event. Although the *UAS-egr*, *GMR-Gal4* and the wildtype second chromosome were not completely isogenic, enough useful FLPs could be identified that are identical in these three stocks but distinguishable from the corresponding FLP on the reference chromosome (EP2L or FRT40). By analyzing flies that still carry the suppressor mutation and flies that lost it (small eyes) the mutation could be approached from the distal and proximal side.

For those suppressors where FLP mapping was applied, the determined FLP interval is given in the Appendix. For the exact chromosomal position of the corresponding FLPs check Berger et al. (2001) and Zipperlen et al. (2005). This approach was successfully applied for the identification of the gene that is affected in complementation group *L1* (see Chapter 4.7).

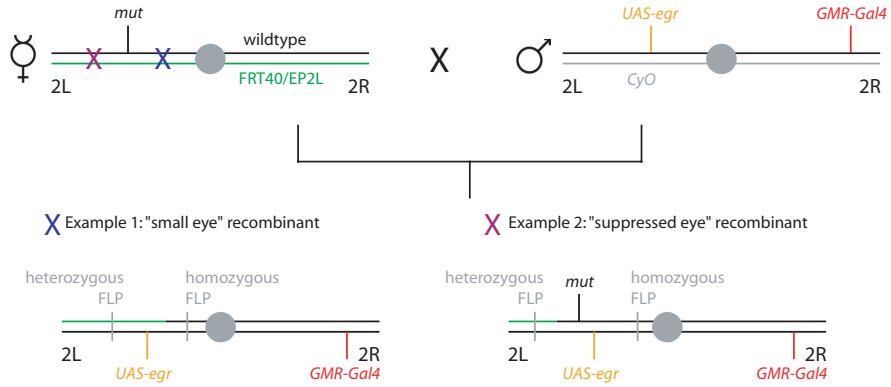
Genetics of FLP mapping

a) Suppressor mutation located on *GMR-Gal4* chromosome (II):



Useful FLPs are identical for *GMR-Gal4* and *UAS-egr* chromosome but different from FRT40/EP2L.

b) Suppressor mutation located on wildtype chromosome (II):



Useful FLPs are identical for *GMR-egr* and wildtype chromosome but different from FRT40/EP2L.

Figure 6 Crossing schemes for generation of recombinants used for FLP mapping.

4.5 Identification and Characterization of *Drosophila* TAB2

Geuking, P., Narasimamurthy R. and Basler K.

A Genetic Screen Targeting the Tumor Necrosis Factor/Eiger Signaling Pathway: Identification of *Drosophila* TAB2 as a Functionally Conserved Component

Genetics **171**: 1683-1694, 2005

In this paper we describe the isolation and characterization of mutations in *bsk*, *dTAK1* and *Drosophila TAB2*. In particular we focus on the description of the screen and the characterization of dTAB2.

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A Genetic Screen Targeting the Tumor Necrosis Factor/Eiger Signaling Pathway: Identification of Drosophila TAB2 as a Functionally Conserved Component

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ABSTRACT

Signaling by tumor necrosis factors (TNFs) plays a prominent role in mammalian development and disease. To fully understand this complex signaling pathway it is important to identify all regulators and transduction components. A single TNF family member, Eiger, is encoded in the Drosophila genome, offering the possibility of applying genetic approaches for pursuing this goal. Here we present a screen for the isolation of novel genes involved in the TNF/Eiger pathway. On the basis of Eiger's ability to potently activate Jun-N-terminal kinase (JNK) and trigger apoptosis, we used the Drosophila eye to establish an assay for dominant suppressors of this activity. In a large-scale screen the Drosophila homolog of TAB2/3 (dTAB2) was identified as an essential component of the Eiger-JNK pathway. Genetic epistasis and biochemical protein-protein interaction assays assign an adaptor role to dTAB2, linking dTRAF1 to the JNKKK dTAK1, demonstrating a conserved mechanism of TNF signal transduction in mammals and Drosophila. Thus, in contrast to morphogenetic processes, such as dorsal closure of the embryo, in which the JNK pathway is activated by the JNKKK Slipper, Eiger uses the dTAB2-dTAK1 module to induce JNK signaling activity.

LIGANDS of the tumor necrosis factor (TNF) family regulate fundamental processes in humans, such as apoptosis, cell survival, differentiation, proliferation, and inflammation. Deregulation of TNF signaling pathways is associated with many diseases, including autoimmune disorders and cancer. The study of TNF signaling mechanisms in mammalian systems is complicated by the existence of numerous ligands and receptors and at least three different intracellular signaling pathways (Wallach *et al.* 1999; Locksley *et al.* 2001). Recently it became apparent that there is a single TNF ligand (Eiger) encoded in the Drosophila genome (Igaki *et al.* 2002; Moreno *et al.* 2002; Kauppila *et al.* 2003), raising the prospect of investigating conserved principles underlying this signaling system by simple genetic means.

Like a subset of the mammalian TNF proteins, Eiger is a potent inducer of apoptosis. Unlike its mammalian counterparts, however, the apoptotic effect of Eiger does not require the activity of the caspase-8 homolog DREDD, but it completely depends on its ability to activate the Jun-N-terminal kinase (JNK) pathway and subsequent activation of the Drosophila apoptosome (DARK + DRONC) (Moreno *et al.* 2002). Although the JNK pathway is used multiple times in Drosophila development, Eiger is the only known extracellular protein that triggers its activation. Of particular interest is therefore the interface between the cell membrane and the core JNK cassette [consisting of the JNKK Hemipterous (Hep), the JNK Basket (Bsk), and the transcription factors Jun and Fos]. Apart from Wengen (Wgn), the presumptive TNF receptor homolog in Drosophila (Kanda *et al.* 2002; Kauppila *et al.* 2003), no other components have been convincingly implicated in JNK activation upstream of the candidate JNKKK dTAK1 (Vidal *et al.* 2001).

Here we designed and performed a genetic screen to isolate rate-limiting components in mediating Eiger-induced apoptosis. We report the identification of >100 mutations that weaken the Eiger-JNK pathway. While some of these mutations affect already known components, such as Bsk and dTAK1, and thus validate the screen, we further show that one group of alleles inactivates a previously uncharacterized Drosophila gene, *CG7417*. By genetic and biochemical means we demonstrate that it functions as the TAB2/3 homolog. TAB2/3 have been demonstrated to link TNF receptor-associated factor (TRAF) proteins to TAK1 in mammalian interleukin- and TNF-signal transduction (Takaesu *et al.* 2000; Ishitani *et al.* 2003). We propose that the Drosophila TAB2/3 homolog provides, together with dTRAF1, an adaptor function enabling the presumptive JNKKKK Misshapen (Msn) to activate the JNKKK dTAK1, which in turn advances the Eiger signal to the JNKK Hep and its JNK substrate Bsk.

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MATERIALS AND METHODS

Fly stocks: The Bloomington Drosophila Stock Center (BDSC) provided *bsk*^{1}, *Df(2R)P34*, and the Exelixis deficiency collection. The following reagents have been described previously: *UAS-egr* (Moreno *et al.* 2002), *dTAK1* alleles and their rescuing transgene (Vidal *et al.* 2001), *UAS-hep*^{CA} (Adachi-Yamada *et al.* 1999), and *GMR-Gal4* (Hay *et al.* 1994).

Stocks carrying the *UAS-Drosophila homolog of TAB2/3* (*dTAB2*) and *tubulinα1-dTAB2* transgene were obtained by standard *P*-element-induced transformation. The *dTAB2* full-length cDNA (LD40663) was cloned into pUAST (Brand and Perrimon 1993) or into a vector containing the *tubulinα1* promoter (Basler and Struhl 1994), respectively.

EMS mutagenesis: Drosophila males carrying the *GMR-Gal4* insertion were starved for 8 hr before mutagenesis. These males were then kept for 24 hr in a bottle containing a filter paper soaked with 0.4% EMS in sugar solution (1 g/100 ml). After a recovery phase of another 24 hr on normal food, the mutagenized males were mated at 25° with virgins carrying the *UAS-egr* transgene.

Genetic distance to *GMR-Gal4* insertion: Chromosomes carrying the *GMR-Gal4* insertion and a suppressor mutation were allowed to recombine with a wild-type chromosome in females. Such virgins were crossed to *UAS-egr* males. The number of *GMR-Gal4* progeny with a suppressed eye phenotype in relation to the number of *GMR-Gal4* progeny with a small eye phenotype reflects the genetic distance between the suppressor mutation and the *GMR-Gal4* insertion.

Generation and analysis of *ey-flp* mosaics: For genes that did not dominantly suppress the Eiger-induced small eye phenotype, such as *djun* and *dTRAF1*, *ey-flp* clones were generated to obtain animals with eyes composed largely of homozygous mutant cells (Newsome *et al.* 2000). In this background the ability of Eiger to induce apoptosis was analyzed. Such analysis was uninformative, however, for the *dTRAF1*^{ex1} allele (Cha *et al.* 2003) as mosaic eyes exhibited a distorted pattern already in the absence of Eiger expression.

Sequencing: Genomic DNA was amplified by PCR using evenly spaced primers in the *CG7417*, *bsk*, and *dTAK1* coding regions. PCR products were analyzed by standard sequencing.

***Drosophila* cell culture and transfection:** Schneider (S2) cells were cultured in Schneider's Drosophila medium (Invitrogen, San Diego) supplemented with 10% fetal calf serum and 1% penicillin/streptomysin at 25°. Cells were transfected with expression vectors, using Cellfectin (Invitrogen) according to the manufacturer's protocol.

Expression vectors: Full-length *dTRAF1* and *dTRAF2* coding sequences were amplified by PCR, using ESTs RE63023 and RE19938 as templates, respectively. These PCR fragments were inserted into the triple-HA-containing vector pMZ55 and subcloned into pUAST along with the 3× HA tag. The *dTAK1*-FLAG construct was amplified by PCR from transgenic flies harboring *UAS-dTAK1* (gift from Makoto Nakamura) and inserted into pUAST. *FLAG-dTAB2*, *HA-dTAB2-N*, *HA-dTAB2-C*, and *HA-dTAB2-Δcc* were amplified by PCR from a *HA-dTAB2* construct and cloned into pUAST. The *UAS-Wengen* plasmid was a gift from E. Moreno.

Immunoprecipitation and immunoblotting: S2 cells (0.75×10^6 cells/well) were seeded into a 12-well plate. One day after seeding cells were transfected with the indicated expression vectors. Forty-eight hours after transfection the cells were harvested and lysed in lysis buffer containing 150 mm NaCl, 50 mm Tris-HCl (pH 8.0), 1% Nonidet P-40, 0.5% deoxycholic acid, and protease inhibitors (Complete Mini; Roche, Indianapolis). Lysates were mixed either with an anti-HA antibody and 25 μl of Protein-A sepharose beads or with 25 μl of anti-FLAG agarose beads (Sigma, St. Louis) and allowed to rotate at 4° overnight. The beads were then collected and washed with the lysis buffer four times. Proteins were eluted from the beads and resolved on a 4–12% NUPAGE gel system (Invitrogen) and transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with either anti-HA antibody (3F10, Roche) or anti-FLAG M2 antibody (Sigma) followed by appropriate secondary antibodies conjugated with horseradish peroxidase (HRP). Signals were detected with ECL reagents (Amersham, Arlington Heights, IL).

Double-stranded RNA production: Double-stranded RNA (dsRNA) was prepared as described by the Dixon lab (Clemens *et al.* 2000). Briefly, using PCR products as templates, the MEGASCRIPT T7 transcription kit (Ambion, Austin, TX) was used to produce RNA according to the manufacturer's protocol. RNA products were ethanol precipitated and resuspended in DEPC-treated water. dsRNA was generated by annealing at 65° for 30 min followed by slow cooling to room temperature. The following sets of forward (FP) and reverse primers (RP) were used: *Basket* (FP 5′ cgccgcaaaggaacttgg 3′; RP 5′ tcagcatcatac cacacg 3′), *dTAK1* (FP 5′ gatgaccaacaatcgcgg 3′; RP 5′ ggcgct gagtggcctcagc 3′), *msn* (FP 5′ atggcgcaccagcagcaacaac 3′; RP 5′ ccatctccagagcggtgatgc 3′), and *dTAB2* (FP 5′ atggcggctacaccac caatgc-3′; RP 5′ gtcgctgctggcgctgcataatc 3′).

LPS treatment: S2 cells were treated with dsRNA (15 μg/10^6 cells) as indicated in Figure 5F. The cells were then split into two. One-half was left untreated and one-half was treated with lipopolysaccharide (LPS) (Sigma) at a concentration of 50 μg/ml for 10 min. The cells were then lysed in lysis buffer. The lysates were analyzed by immunoblotting to detect phosphorylated JNK (Promega, Madison, WI) and JNK (Santa Cruz Biotechnologies, Santa Cruz, CA).

Luciferase assay: S2 cells (0.4×10^6 cells/well) were seeded into a 24-well plate. One day after seeding cells were transfected with an AP1-luciferase reporter plasmid along with the indicated expression vector. The total DNA concentration (1 μg) was kept constant by supplementing with empty vector. Forty-eight hours after transfection, cells were harvested, lysed in passive lysis buffer, and luciferase activity was measured using the dual luciferase assay system (Promega). The values shown reflect the relative luciferase activity: the ratio of firefly (AP1 luciferase) and *tub-renilla* luciferase activity of one representative experiment in which each transfection was made in duplicate.

RESULTS

A dominant modifier screen to identify new components of the Eiger-JNK pathway: Forced expression of Eiger in the developing compound eye of Drosophila triggers massive apoptosis and results in a small eye phenotype (Figure 1, A and B; Igaki *et al.* 2002; Moreno *et al.* 2002; Kauppila *et al.* 2003). A reduction in copy number of genes encoding core JNK pathway components partially rescues this phenotype (Figure 1C; Igaki *et al.* 2002; Moreno *et al.* 2002). Complete elimination of *bsk* (encoding Drosophila JNK) or *djun* in mosaic animals by genetic means or suppression of Bsk activity by forced expression of the JNK-phosphatase Puckered (Martin-Blanco *et al.* 1998) reverted the Eiger-induced small eye phenotype (data not shown and Moreno *et al.* 2002). Hence, all apoptosis-inducing activity of the Eiger pathway is apparently transduced by the JNK pathway (Figure 1E). Animals heterozygous for *dTAK1* dominant-negative alleles (Figure 1D) or hemizygous

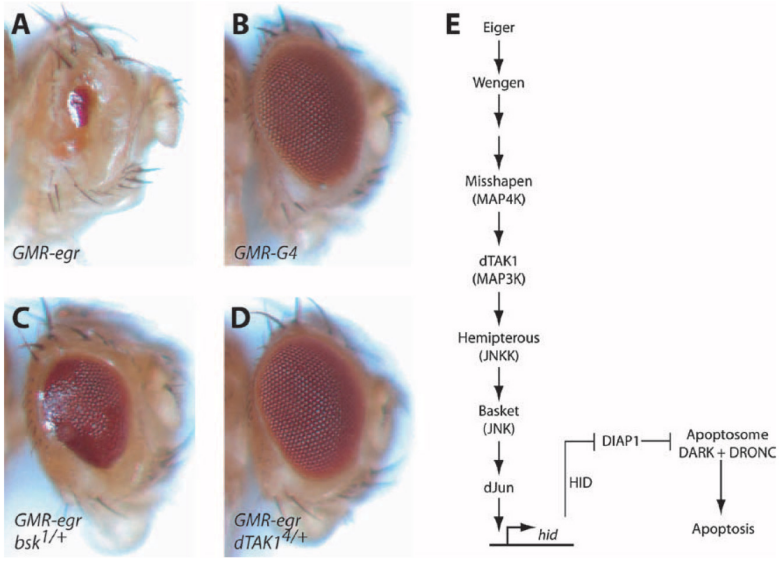

FIGURE 1.—The small eye phenotype caused by *eiger (egr)* overexpression in the eye provides a sensitized system to screen for new components. (A) *GMR-Gal4* UAS-*egr*/+. Overexpression of *egr* in the Drosophila compound eye leads to a small eye phenotype due to massive induction of apoptosis. (B) *GMR-Gal4*/+ control eye. (C) *GMR-Gal4 UAS-egr*/*bsk*^{1}. Removing a single copy of the Drosophila JNK gene *bsk* dominantly suppresses the small eye phenotype. (D) *dTAK1*^{4}/+ ; *GMR-Gal4 UAS-egr*/+. *dTAK1*^{4} is a dominant-negative allele of *dTAK1*, which fully suppresses the small eye phenotype in a dominant fashion. (E) Schematic representation of the current model of Eiger signaling.

mutant for *dTAK1* (null allele, Figure 4F) also show a complete suppression of the small eye phenotype. Thus dTAK1 appears to provide the most relevant JNKKK function in the Eiger pathway, as none of the other five putative JNKKK homologs encoded in the Drosophila genome (STRONACH 2005) can substitute for dTAK1. Indeed, removing one copy of *slipper*, which codes for a JNKKK involved in JNK activation during the morphogenetic process of dorsal closure (STRONACH and PERRIMON 2002), does not suppress the small eye phenotype (data not shown).

The above described assay was used as a basis for a screening system to identify genes required for Eiger signaling. Adult males carrying a *GMR-Gal4* transgene were mutagenized with EMS and mated to *UAS-eiger* females (*UAS-egr*; see crossing scheme in Figure 2A). The progeny was scored for suppression of the small eye phenotype. Candidate suppressors were isolated, retested, and mapped to individual chromosomes by virtue of visible markers on chromosomes 2 and 3 (Figure 2A). After screening ~55,000 animals, 117 stocks with suppressor mutations were established (Figure 2B), each categorized in one of three phenotypic classes on the basis of the extent of the rescue: "complete" (not shown), "intermediate" (Figure 2C), and "weak" (Figure 2D).

Validation of the screen: In our screen, the genome of GMR-Gal4 animals was exposed to the EMS mutagen. Since Gal4 activity is vital for the small eye phenotype, we expected some of the suppressors to harbor mutations in the *Gal4* driver transgene. Twenty-one such events were indeed identified on the basis of the following criteria: (i) lack of recombination separating the suppressor mutation and the *GMR-Gal4* transgene insertion and (ii) suppression of unrelated Gal4-dependent overexpression phenotypes, such as the *UAS-Inr*-driven big eye phenotype (BROGIOLO *et al.* 2001). Most members of this class show a full reversion of the Eiger-induced small eye phenotype and thus accounted for the vast majority of the complete suppressors.

As described above, JNK activity is critical for the transduction of the Eiger signal. One prediction for our screen would therefore be that it leads to the isolation of new *basket* (*bsk*) alleles (Figure 2E). All suppressor mutations that mapped to the second chromosome were subjected to a complementation analysis with a previously described allele of *bsk* (RIESGO-ESCOVAR *et al.* 1996; SLUSS *et al.* 1996). Ten mutations failed to complement *bsk*^{1} and subsequent sequence analysis revealed that all of them carry molecular lesions in the *bsk* coding region (Figure 2J, Table 1).

Only one mutation (*G14*) mapped to the X chromosome (Figure 2F). This mutation is homozygous viable and completely suppresses the Eiger-induced small eye phenotype when hemizygous (not shown). Since this behavior reflects exactly that of known *dTAK1* alleles (Figure 4F), we expected, and also found, a mutation in the *dTAK1* coding region of *G14*. To confirm that the detected mutation (Thr221 → Iso) is indeed responsible for the observed suppression of Eiger signaling, rescue experiments with a genomic *dTAK1* transgene were performed (VIDAL *et al.* 2001). *G14* animals carrying the *dTAK1* rescue construct displayed a reduced suppression of the small eye phenotype (Figure 2G). The known *dTAK1* allele *dTAK1*^{4} shows the same behavior (Figure 2H, compare with Figure 1D). The presumed dominant-negative nature of these two *dTAK1* alleles (*4* and *G14*) may explain why the eye phenotype is not completely reverted to "small." Indeed, the suppression activity of a presumed *null* allele of *dTAK1* is fully inhibited by the *dTAK1* transgene rescue construct

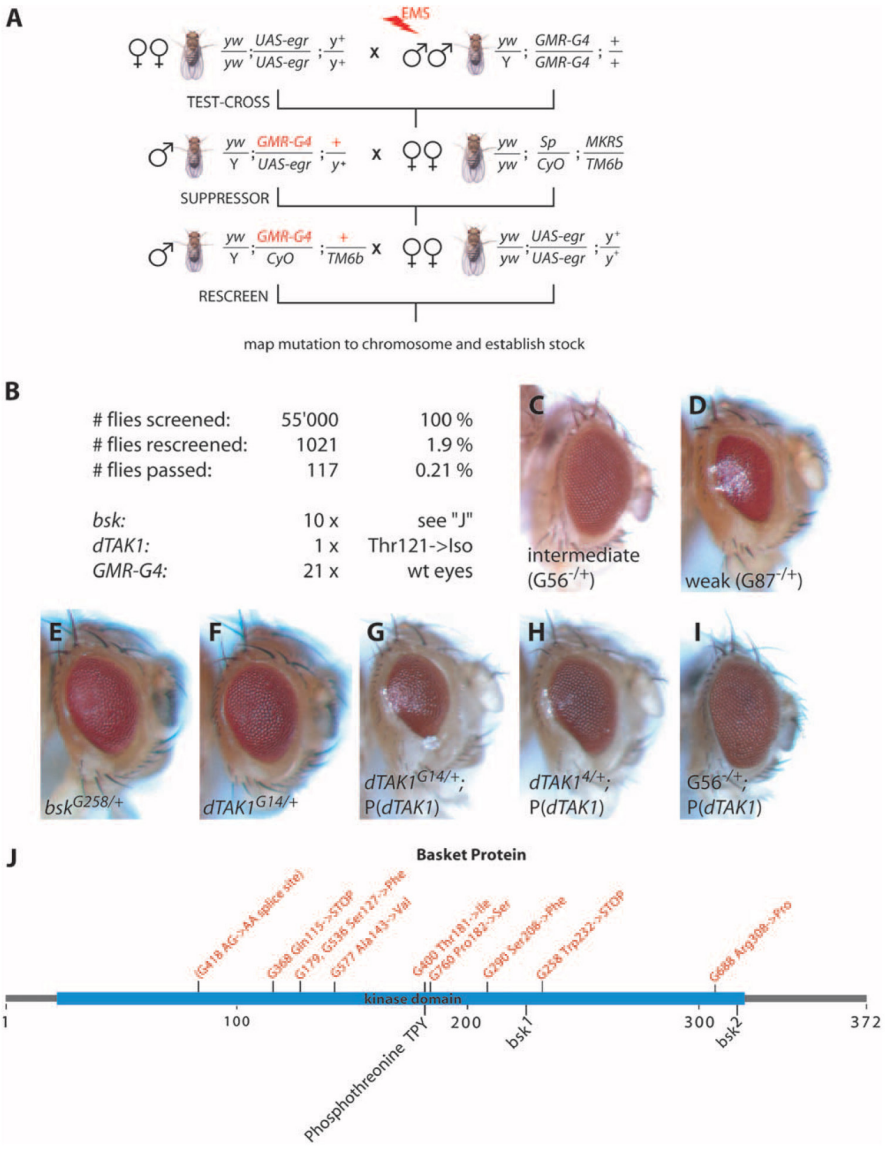

FIGURE 2.—A dominant modifier screen to identify new components of the Eiger pathway in Drosophila. (A) Crossing scheme. Males carrying a *GMR-Gal4* transgene are mutagenized with EMS and crossed to virgins carrying a *UAS-egr* transgene. Suppressors were rescreened, balanced, and mapped to a chromosome by virtue of visible dominant markers. The second chromosome is marked by the w^+ (dark red) included in the *GMR-Gal4* insertion. The w^+ of the *UAS-egr* insertion displays an orange eye color. The third chromosome is marked by the absence of a P[y^+] insertion present on the non-EMS-treated chromosome. Lethal mutations on the X chromosome escaped our detecting system. Since the X chromosome was not marked, viable mutations, which were obtained only when female suppressors were selected, could not be followed and therefore were obtained only rarely. (B) Numbers of animals that were screened and rescreened and stocks that were established, with the number of alleles identified for positive controls. C–I are in a *GMR-Gal4 UAS-egr*/+ background. (C) *GMR-Gal4 UAS-egr*/*G56*. An example of a suppressor belonging to the "intermediate" class is shown. (D) *GMR-Gal4 UAS-egr*/*G87*. An example of a suppressor belonging to the "weak" class is shown. (E) *GMR-Gal4 UAS-egr*/bsk^{G258}. *G258*, one of the new *bsk* alleles identified in the screen, dominantly suppresses the small eye phenotype. (F) $dTAK1^{G14}$/+; *GMR-Gal4 UAS-egr*/+. *G14*, the *dTAK1* allele identified in the screen, dominantly suppresses the small eye phenotype. (G) $dTAK1^{G14}$/+; *GMR-Gal4 UAS-egr*; *P(dTAK1)*/+. (H) $dTAK1^{4}$/+; *GMR-Gal4 UAS-egr*/+; *P(dTAK1)*/+. (I) *GMR-Gal4* UAS-*egr*/*G56*; *P(dTAK1)*/+. A *dTAK1* genomic rescue transgene partially reverts the suppression of the small eye phenotype caused by heterozygous loss of $dTAK1^{G14}$ or $dTAK1^{4}$ but not the suppression caused by the unrelated suppressor mutation *G56*. The suppression of the small eye phenotype is not completely reverted by the *dTAK1* rescue construct due to the dominant-negative nature of the $dTAK1^{G14}$ and $dTAK1^{4}$ alleles. Both carry amino acid substitutions in the kinase domain of dTAK1 (VIDAL *et al.* 2001), which leads to dominant-negative proteins. (J) Molecular lesions found in the 10 novel *bsk* mutants.

(Figure 4G), while an unrelated suppressor mutation (*G56*) from our screen showed the same extent of suppression irrespective of the presence or absence of the *dTAK1* rescue construct (Figure 2I, compare with Figure 2C).

Mapping and molecular cloning of a novel Eiger suppressor: Complementation analysis revealed that, with the exception of our *bsk* alleles and one thus far uncharacterized complementation group, most of the mutations isolated in our screen are homozygous viable like mutations in the *dTAK1* or *eiger* (*egr*) genes (VIDAL *et al.* 2001; IGAKI *et al.* 2002). To group our second chromosomal suppressors by other means, we mapped them by recombination analysis relative to the *GMR-Gal4* insertion (see MATERIALS AND METHODS). In parallel, we screened a large collection of deficiencies for dominant suppressors of the Eiger-induced small eye phenotype. These deficiencies are molecularly mapped and uncover ~56% of the Drosophila genome (PARKS *et al.* 2004). Interestingly, one group of our EMS-induced mutations mapped to the same chromosomal region as deficiency *Df(2R)Exel6069*, which behaved as a suppressor

TABLE 1

Molecular lesions identified in *basket* (*bsk*)

Allele	DNA level	Protein level
G418	3′ splice site intron 3 A**G** → A**A**	—
G368	**C**AG → **T**AG	Gln115 → Stop
G179 (independent of G536)	T**C**C → T**T**C	Ser127 → Phe
G536 (independent of G179)	T**C**C → T**T**C	Ser127 → Phe
G577	G**C**A → G**T**A	Ala143 → Val
G400	A**C**C → A**T**C	Thr181 → Ile
G760	**C**CC → **T**CC	Pro182 → Ser
G290	T**C**C → T**T**C	Ser208 → Phe
G258	TG**G** → TG**A**	Trp232 → Stop
G688	C**G**G → C**C**G	Arg308 → Pro

Alleles are ordered according to their position in the protein. Nucleotide changes are in bold face type. In a *GMR-Gal4 UAS-egr* background, all alleles display an "intermediate" strength of suppression.

of Eiger signaling (Figure 3B). *Df(2R)Exel6069* uncovers only 20 genes at cytological position 56B5–56C11. By using overlapping deficiencies and sequence analysis (Figure 3A), we identified in 39 suppressors molecular lesions in gene *CG7417* (Figure 3C, Table 2). We interpreted these results as an indication that *CG7417* may encode a component critically required for Eiger signaling.

CG7417 encodes the Drosophila homolog of TAB2: The full open reading frame (represented by cDNA *LD40663*) of *CG7417* encoded an uncharacterized protein of 831 amino acids with a CUE, a coiled-coil, and a zinc-finger domain (Figure 3C). These domains are found together only in human TAB2 and TAB3 and in homologs of these proteins in other organisms. Human TAB2 and TAB3 are almost identical and were identified as binding partners of TAK1 (Takaesu *et al.* 2000; Ishitani *et al.* 2003; Cheung *et al.* 2004; Jin *et al.* 2004). On the basis of the conserved domain architecture we propose CG7417 as the Drosophila homolog of TAB2 and TAB3 and hereafter refer to *CG7417* as *dTAB2*.

Each of the 39 *dTAB2* alleles displays a similar degree of suppression of the Eiger-induced small eye phenotype (Figure 4A). Removing both copies of *dTAB2* does not fully revert the small eye phenotype to wild type (Figure 4B), indicating that even in complete absence of dTAB2 a slight activation of the pathway can occur (see discussion). To verify that the suppression activity of our alleles is indeed caused by the mutations detected in

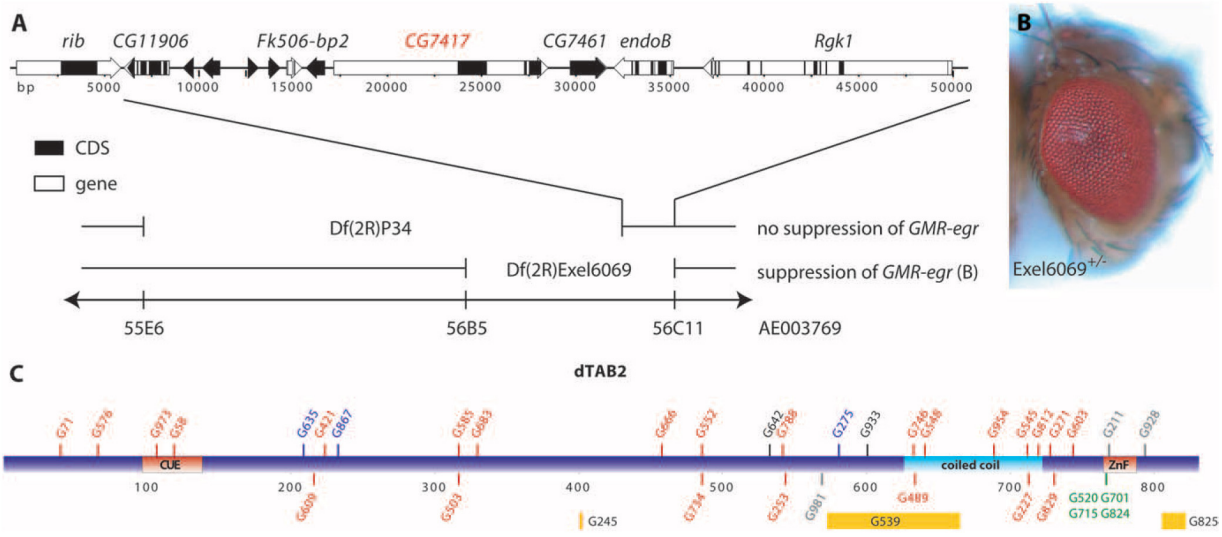

Figure 3.—Identification of *dTAB2 (CG7417)*. (A) The two overlapping deficiencies *Df(2R)Exel6069* and *Df(2R)P34* narrow down the region of interest to 11 genes. The distal breakpoint of *Df(2R)P34* was placed between *CG11906* and *ribbon* on the basis of the fact that *Df(2R)P34* and *ribbon* fail to complement each other (Bradley and Andrew 2001). The *CG7417* coding region has a mutation in 39 of the established suppressor stocks. (B) *GMR-Gal4 UAS-egr/Df(2R)Exel6069*. *Df(2R)Exel6069* dominantly suppresses the small eye phenotype whereas *Df(2R)P34* does not. (C) Domain architecture and mutations identified in the dTAB2 protein. Red, Gln → Stop. Blue, Arg → Stop. Green, Trp → Stop. Gold, deletions. Black, splice sites mutated. Gray, amino acid substitutions.

TABLE 2

Molecular lesions identified in *dTAB2* (*CG7417*)

Allele	DNA level	Protein level
G71	**C**AG → **T**AG	Gln40 → Stop
G576	**C**AG → **T**AG	Gln66 → Stop
G973	**C**AG → **T**AG	Gln107 → Stop
G58	**C**AA → **T**AA	Gln119 → Stop
G635	**C**GA → **T**GA	Arg209 → Stop
G609	**C**AA → **T**AA	Gln216 → Stop
G421	**C**AG → **T**AG	Gln224 → Stop
G867	**C**GA → **T**GA	Arg233 → Stop
G503 (independent of G585)	**C**AA → **T**AA	Gln317 → Stop
G585 (independent of G503)	**C**AA → **T**AA	Gln317 → Stop
G683	**C**AG → **T**AG	Gln330 → Stop
G245	7-bp Del bp 1203–1209 of CDS	Premature Stop at AA422
G666	**C**AG → **T**AG	Gln458 → Stop
G552 (independent of G734)	**C**AG → **T**AG	Gln486 → Stop
G734 (independent of G552)	**C**AG → **T**AG	Gln486 → Stop
G642	5′ splice site intron 2 G**T** → G**A**	—
G788	**C**AG → **T**AG	Gln542 → Stop
G253	**C**AG → **T**AG	Gln544 → Stop
G981	C**T**G → C**A**G + **G**AC → **A**AC	Leu570 → Gln + Asp592 → Asn
G539	284-bp Del bp 1711–1994 of CDS + CA insertion at breakpoint → in frame!	Thr571 → His, Glu572 → Gln, AA 573–666 deleted
G275	**A**GA → **T**GA	Arg581 → Stop
G933	3′ splice site intron 3 A**G** → A**A**	—
G746	**C**AA → **T**AA	Gln633 → Stop
G489	**C**AG → **T**AG	Gln634 → Stop
G548	**C**AG → **T**AG	Gln641 → Stop
G954	**C**AG → **T**AG	Gln689 → Stop
G545	**C**AG → **T**AG	Gln712 → Stop
G227	**C**AG → **T**AG	Gln713 → Stop
G812	**C**AG → **T**AG	Gln720 → Stop
G271	**C**AG → **T**AG	Gln728 → Stop
G829	**C**AG → **T**AG	Gln731 → Stop
G603	**C**AG → **T**AG	Gln744 → Stop
G520 (independent of G715)	T**G**G → T**A**G	Trp767 → Stop
G715 (independent of G520)	T**G**G → T**A**G	Trp767 → Stop
G701 (independent of G824)	TG**G** → TG**A**	Trp767 → Stop
G824 (independent of G701)	TG**G** → TG**A**	Trp767 → Stop
G211	**T**GC → **G**GC + AA**C** → AA**A**	Cys769 → Gly + Asn770 → Cys
G928	G**G**T → G**A**T	Gly794 → Asp
G825	53-bp Del bp 2437–2489 of CDS	Frameshift leads to a longer protein

Alleles are ordered according to their position in the protein. Nucleotide changes are in boldface type. In a *GMR-Gal4 UAS-egr* background, all alleles display an "intermediate" strength of suppression.

the *dTAB2* coding region, rescue experiments with a *tubulinα1* promoter-driven *dTAB2* transgene were carried out. The predicted suppression caused by heterozygosity for *dTAB2* could be overcome by expression of a *tubulinα1-dTAB2* transgene (Figure 4C). Importantly, the *tubulinα1-dTAB2* construct had no effect on the suppression of the Eiger eye phenotype brought about by an unrelated suppressor mutation (*G56*, Figure 4D).

dTAB2 functions upstream of Hep and dTAK1: Expression of a constitutively active form of Hep (hep^{CA}) (Adachi-Yamada *et al.* 1999) causes a reduction in eye size (Figure 4H). This effect is mediated by JNK activation as it is completely inhibited by coexpression of Puc (not shown). The Hep^{CA} small eye phenotype is suppressed by reducing *bsk* activity (Figure 4I), but not by reducing *dTAB2* activity (Figure 4J), indicating that Bsk and dTAB2 act downstream and upstream of Hep, respectively. The Hep^{CA} phenotype is not suppressed in males hemizygous for *dTAK1*, placing dTAK1, like dTAB2, upstream of Hep (not shown). To address where dTAB2 and dTAK1 act relative to each other, epistasis experiments were performed in S2 cells, using a JNK luciferase

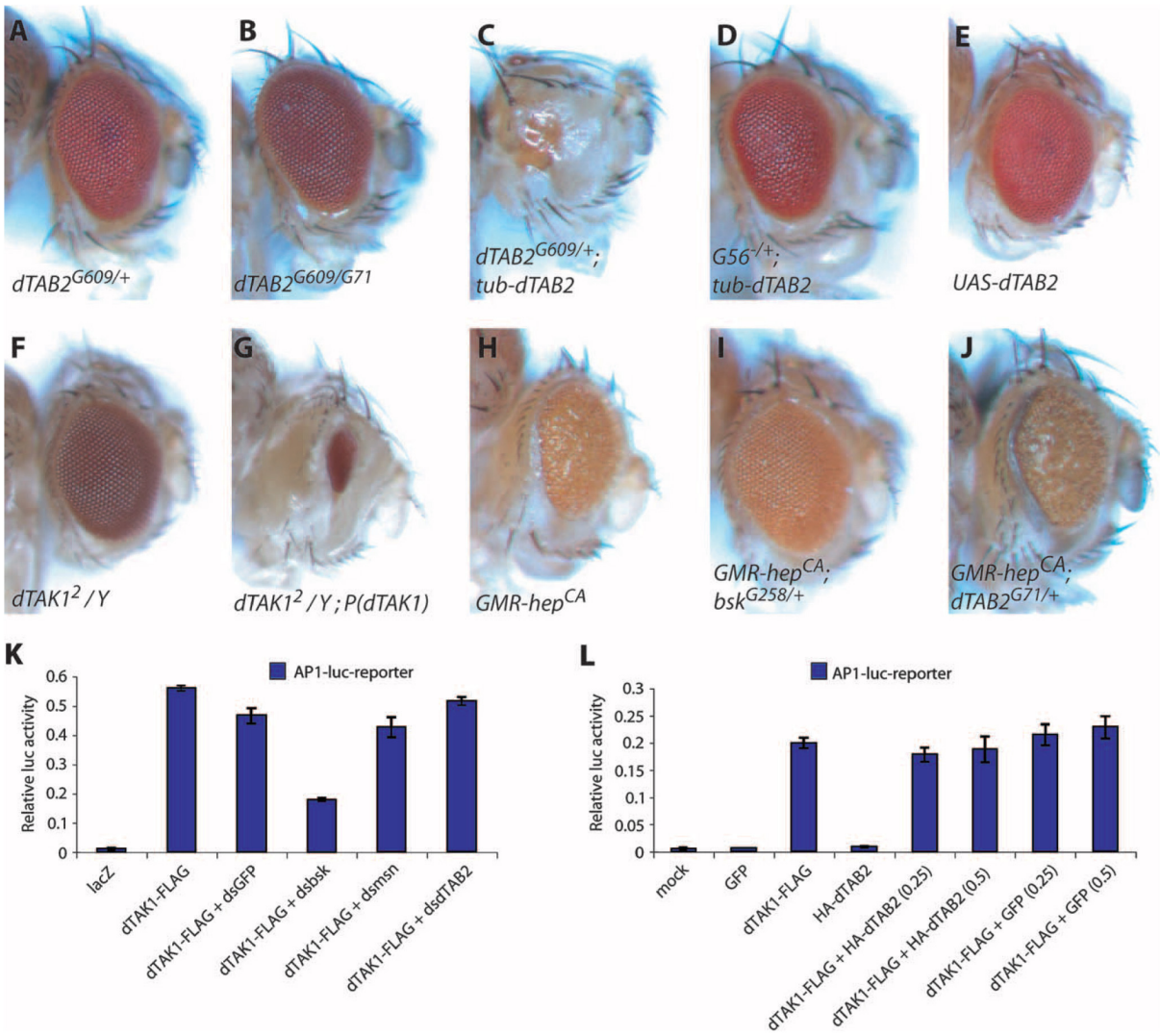

FIGURE 4.—dTAB2 functions upstream of Hep and dTAK1. A–G are in *GMR-Gal4 UAS-egr/+* background. (A) *GMR-Gal4 UAS-egr/dTAB2^{G609}. dTAB2* alleles dominantly suppress the small eye phenotype. (B) *GMR-Gal4 UAS-egr dTAB2^{G609}/dTAB2^{G71}*. The small eye phenotype is not completely suppressed in *dTAB2* homozygous mutant flies. (C) *GMR-Gal4 UAS-egr/dTAB2^{G609}; tub-dTAB2*. (D) *GMR-Gal4 UAS-egr/G56; tub-dTAB2*. A *tub-dTAB2* rescue transgene reverts the suppression of the small eye phenotype caused by heterozygous loss of *dTAB2*, but not the suppression brought about by the unrelated suppressor mutation *G56*. (E) *GMR-Gal4 UAS-egr/UAS-dTAB2*. Overexpression of wild-type *dTAB2* has a dominant-negative effect on Eiger signal transduction. (F) *dTAK1^{2}/Y; GMR-Gal4 UAS-egr/+*. A hemizygous null allele of *dTAK1* completely suppresses the small eye phenotype. *dTAK1^{2}* has an early stop mutation (VIDAL *et al.* 2001). (G) *dTAK1^{2}/Y; GMR-Gal4 UAS-egr/+; P(dTAK1)/+*. In contrast to dominant-negative alleles of *dTAK1* (*G14, 4*), the suppression of the small eye phenotype brought about by a null allele of *dTAK1* can be completely reverted by introducing a *dTAK1* genomic rescue transgene. (H) *GMR-Gal4 UAS-hep^{CA}/+*. (I) *GMR-Gal4* UAS-*hep^{CA}/bsk^{G258}*. (J) *GMR-Gal4 UAS-hep^{CA}/dTAB2^{G71}*. The small eye phenotype caused by overexpression of a constitutive active form of Hep is dominantly suppressed by removing one copy of *bsk* but not by removing one copy of *dTAB2*. (K) RNAi against *bsk* but not against *msn* or *dTAB2* suppresses the dTAK1-FLAG-induced activation of the AP1-luc-reporter in S2 cells. (L) Overexpression of *UAS-HA-dTAB2* does not activate the AP1-luc-reporter. Wild-type dTAB2 does not exert a dominant-negative effect on dTAK1-mediated activation of the JNK pathway. Numbers in parentheses indicate amounts of plasmid in micrograms.

reporter system in combination with RNAi. Expression of dTAK1 strongly activated a JNK luciferase reporter. Reporter activity was reduced by RNAi against *bsk* (Figure 4K) or *djun* (not shown), but not by RNAi against *msn* or *dTAB2* (Figure 4K), although RNAi against *msn* and *dTAB2* strongly reduces their protein levels (data not shown). These experiments place Msn and dTAB2 upstream of dTAK1.

dTAB2 links dTRAF1 to dTAK1: In contrast to Hep^{CA} and dTak1, overexpression of dTAB2—either in S2 cells

(Figure 4L) or *in vivo* (not shown)—does not activate the JNK pathway, indicating that dTAB2 does not function as a direct activator of dTAK1 but possibly provides an adaptor function. Consistent with this notion, we find that overexpression of dTAB2 exerts a dominant-negative effect on Eiger signal transduction, as it suppresses the Eiger-induced small eye phenotype (Figure 4E).

To explore the molecular nature of such an adaptor function we carried out protein-protein interaction assays with candidate partners of dTAB2. We first found that N-terminally HA-tagged dTAB2 can immunoprecipitate C-terminally FLAG-tagged dTAK1 from Drosophila S2 cell lysates, and vice versa (Figure 5A). The N-terminal half of dTAB2 (aa 1–450), which includes the CUE domain, did not bind to dTAK1, but the C-terminal half (aa 451–831), which includes coiled-coil and Zn-finger domains, was sufficient to interact with dTAK1 (Figure 5B). Removal of amino acids 451–749 severely impaired its interaction with dTAK1 (Figure 5B). This indicates that dTAB2, like TAB2/3 in mammalian systems, interacts with dTAK1 most likely through its coiled-coil domain.

On the basis of the proximal placement of dTAB2 in the Eiger pathway (see above), we also analyzed its interaction with the Drosophila homologs of the TRAF proteins. FLAG-dTAB2 was coexpressed either with an HA-dTRAF1 or with HA-dTRAF2 in S2 cells and was immunoprecipitated with an anti-HA antibody. Western blot analysis of the immune complexes with an anti-FLAG antibody revealed that dTRAF1 (the homolog of hTRAF2) and dTRAF2 (the homolog of hTRAF6) precipitated dTAB2 (Figure 5C). The weaker binding of dTAB2 to dTRAF1 compared to dTRAF2 might be explained by the lack of a RING-finger domain in dTRAF1. The interaction of TAB2/3 with TRAF2/6 in mammals is dependent in part on ubiquitination, which is mediated by the ring-finger domain (Takaesu *et al.* 2000; Ishitani *et al.* 2003; Kanayama *et al.* 2004). The intact ZnF domain of TAB2/3 is required for binding to polyubiquitin chains (Kanayama *et al.* 2004).

We also tested the binding of the presumptive Eiger receptor Wengen for its interaction with Drosophila TRAF proteins. Wengen was expressed with either HA-dTRAF1 or HA-dTRAF2 in S2 cells. The dTRAFs were precipitated with anti-HA and the precipitates were analyzed with an anti-Wengen antibody (Figure 5D). In agreement with the result of Kauppila *et al.* (2003) we found that Wengen can interact with dTRAF2. In addition, we find that Wengen also interacts with dTRAF1 (Figure 5D).

Next, we asked whether dTRAF1 interacts with dTAK1 directly or via dTAB2. S2 cells were cotransfected with dTAK1-FLAG and either HA-dTRAF1 or HA-dTRAF2. We could detect a very weak binding of both TRAFs with dTAK1, perhaps mediated by endogenous dTAB2. Upon coexpression of dTAB2, significantly increased amount of dTAK1 was precipitated (Figure 5E). This result suggests that dTAB2 can act as an adaptor molecule to link dTRAFs to dTAK1.

dTAB2 mediates JNK activation also upon LPS stimulation: LPS-induced JNK phosphorylation reflects JNK activation during an innate immune response (Sluss *et al.* 1996; Boutros *et al.* 2002). Treatment of S2 cells with LPS indeed dramatically increases JNK phosphorylation (Figure 4F). RNAi targeting *dTAK1* or *dTAB2* (Figure 4F), but not *eiger*, *wengen*, or *msn* (not shown), prevents this increase in JNK phosphorylation. From this we conclude that dTAB2 mediates dTAK1 activation not only in the Eiger pathway but also in response to other stimuli. Furthermore these results suggest that dTAB2, like dTAK1 (Vidal *et al.* 2001), may also play an important role in innate immunity.

DISCUSSION

Here we describe an effective genetic modifier screen for the identification of components of the primordial TNF-JNK-pathway in Drosophila. The isolation of mutations in *bsk* and *dTAK1* validated the specificity of the screen. In addition, the identification of *dTAB2* alleles demonstrates that this screening system will also lead to the discovery of other novel components, which so far have escaped detection by genetic means. Together with the low redundancy of its genome, our findings indicate that Drosophila serves as a suitable system to genetically dissect the TNF pathway. Identification of new evolutionarily conserved components of the TNF pathway may shed light on as-yet unknown aspects of this signaling system that plays numerous roles in human disease.

The screen: The high number of alleles identified for *bsk* and *dTAB2* suggests that we have reached saturation for dosage-sensitive components, at least for the second and third chromosome. The allele frequencies for the loci analyzed differ considerably. The fact that we found only a single *dTAK1* allele can be explained by the genetic setup, in which only a small fraction of mutagenized X chromosomes are recovered (see Figure 2A legend). While the allele frequencies for the *bsk* and *Gal4* genes are roughly proportional to the size of their coding regions (*bsk*, 1 kb—10 alleles; *Gal4*, 2.6 kb—21 alleles), we isolated a surprisingly high number of *dTAB2* alleles (the *dTAB2* coding region is ~2.5 kb, with 39 molecularly confirmed alleles). This high number of *dTAB2* alleles is particularly surprising when the low degree of sequence conservation is taken into consideration. Only short domains with sequence similarities to its mammalian homologs can be identified (Figure 3C: CUE, coiled coil, and ZnF), consistent with a role as an adaptor protein. The most effective way to abolish the function of an adaptor is to disconnect the two protein interaction domains, which genetically is best achieved by the introduction of a stop codon between these domains. EMS induces primarily G/C → A/T mutations (Ashburner 1989). Codons of only three

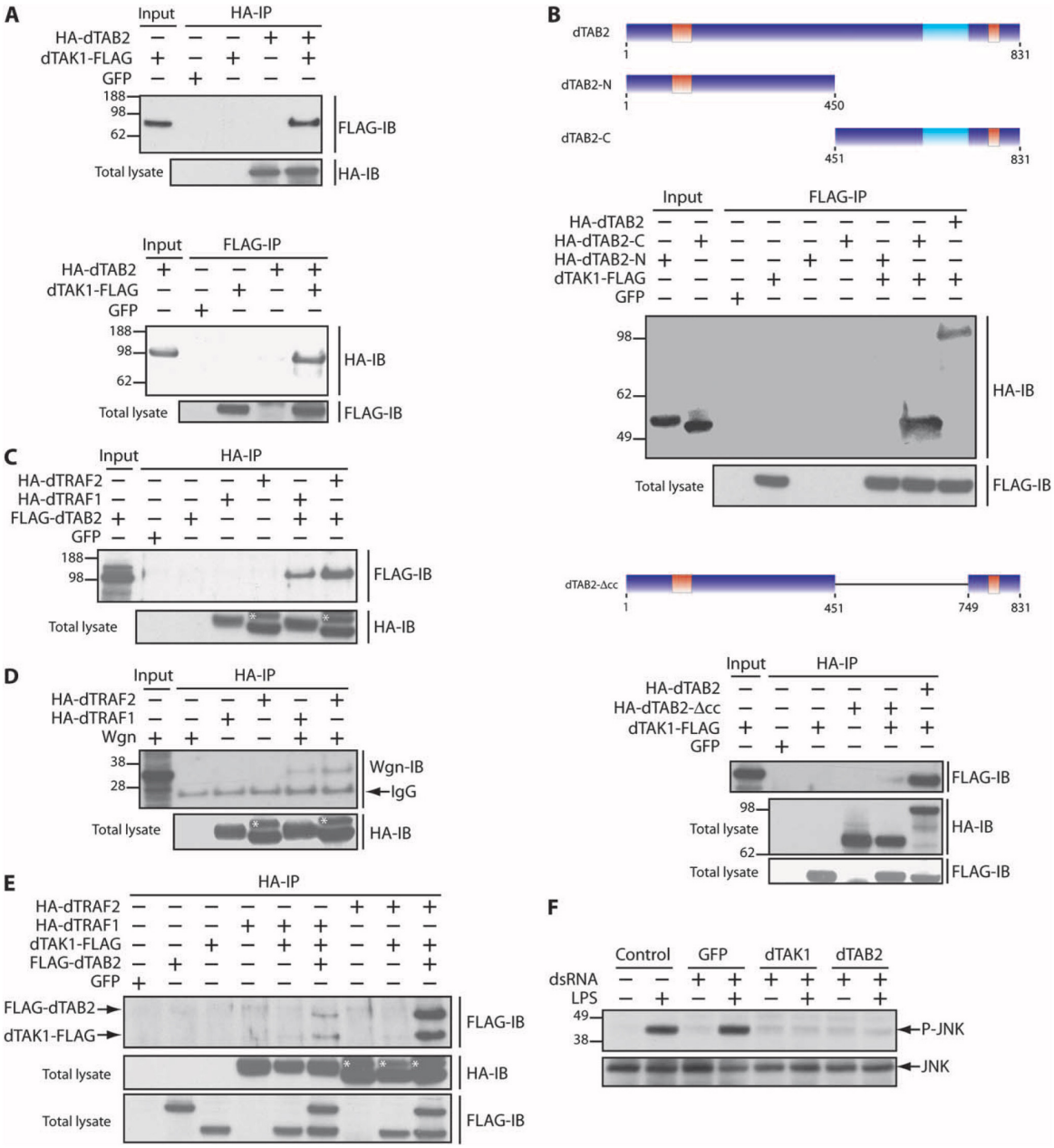

FIGURE 5.—dTAB2 is in a complex with dTRAF1/2 and dTAK1. (A) dTAB2 interacts with dTAK1. S2 cells were transfected with plasmids encoding *UAS-HA-dTAB2* and *UAS-dTAK1-FLAG* together with *ptub-GAL4*. Samples that immunoprecipitated with anti-FLAG antibody were immunoblotted with anti-HA antibody and vice versa. (B) dTAB2 interacts with dTAK1 most likely through its coiled-coil domain. S2 cells were transfected with plasmids encoding *UAS-dTAK1-FLAG* and *UAS-HA-dTAB2* or *UAS-HA-dTAB2-N* or *UAS-HA-dTAB2-C* or *UAS-HA-dTAB2-Δcc* together with *ptub-GAL4*. Anti-FLAG immunoprecipitated samples were immunoblotted with anti-HA antibody or vice versa. (C) dTAB2 associates with both dTRAF1 and dTRAF2. S2 cells were transfected with plasmids encoding *UAS-FLAG-dTAB2* and *UAS-HA-dTRAF1* or *UAS-HA-dTRAF2* together with *ptub-GAL4*. Samples immunoprecipitated with the anti-HA antibody were detected with anti-FLAG antibody. (D) The presumptive Eiger receptor Wengen interacts with both dTRAF1 and dTRAF2. S2 cells were transfected with plasmids encoding Wengen and *UAS-HA-dTRAF1* or *UAS-HA-dTRAF2* together with *ptub-GAL4*. Anti-HA antibody immunoprecipitated samples were immunoblotted with an anti-Wengen antibody. (E) dTAB2 links dTRAF1 and dTRAF2 to dTAK1 and forms a triple complex. S2 cells were transfected with plasmids encoding *UAS-FLAG-dTAB2*, *UAS-dTAK1-FLAG*, and *UAS-HA-dTRAF1* or *UAS-HA-dTRAF2* together with *ptub-GAL4*. Anti-HA antibody-immunoprecipitated samples were immunoblotted with anti-FLAG antibody. (C–E) Asterisks (*) indicate nonspecific bands in the HA-dTRAF2 lysates. (F) dTAB2 is also required for LPS-induced JNK activation. RNAi against dTAK1 or dTAB2 but not against GFP abolishes LPS-mediated phosphorylation of JNK in S2 cells.

amino acids can be mutated to stops by this means: Gln (CAA, CAG), Trp (TGG), and Arg (only CGA). For Arg five other codons exist for which this is not the case, while all Gln and Trp codons can serve as substrates for EMS-induced nonsense mutations. Thus the frequency by which EMS causes premature chain terminations in a gene is largely a function of the Gln and Trp content of its product. It is interesting to note, therefore, that dTAB2 has a Gln content that exceeds the mean Gln frequency of the Drosophila proteome by more than a factor of 2.5 (13.7% *vs.* 5.1%). Indeed, molecular analysis of our *dTAB2* alleles revealed that 24 of the 39 alleles are nonsense mutations of Gln codons (Figure 3C, red alleles).

The pathway: A central issue concerning the TNF/JNK pathway relates to the question of how TAK1 is activated (Shibuya *et al.* 1996; Kishimoto *et al.* 2000; Sakurai *et al.* 2000). On the basis of previous studies and our genetic and biochemical analysis we propose a model for the Eiger pathway, in which dTAB2 and dTRAF1 function as adaptors between the JNKKKK Msn and the JNKKK dTAK1 and in this way may mediate activation of dTAK1 by Msn and the subsequent transduction of the signal via Hep and Bsk (Figure 6). The outline of the pathway is based on the following arguments:

1. Genetic studies have demonstrated the involvement of Msn and dTAK1 in Eiger signaling (Igaki *et al.* 2002; Moreno *et al.* 2002).
2. We identified dTAB2 as an additional component of the Eiger pathway (this article).
3. Epistasis experiments in S2 cells and *in vivo* place Msn and dTAB2 upstream of dTAK1 and Hep (Figure 4, H–K).
4. Liu *et al.* (1999) have shown that Msn interacts with dTRAF1.
5. dTAB2 also binds to dTRAF1 (Figure 5C). Although we failed to detect biochemical evidence for a triple complex Msn-dTRAF1-dTAB2 (data not shown), points 4 and 5 suggest that dTRAF1 may act as an adaptor to link Msn and dTAB2. It is possible that such a complex forms only transiently and is thus difficult to detect biochemically.
6. A triple complex consisting of dTRAF1-dTAB2-dTAK1 can form (Figure 5D), in which dTAB2 functions as a link between dTRAF1 and dTAK1. While each of the above arguments may also be compatible with other models, they collectively support a scenario (Figure 6) in which dTAB2 facilitates the phosphorylation of dTAK1 by Msn. The dominant-negative effect observed by expression of wild-type dTAB2 is an indication that dTAB2 protein levels are critical for proper complex formation (Figure 4E). Our observation that the Eiger-induced small eye phenotype is not entirely suppressed in animals homozygous mutant for dTAB2 suggests that even in the absence of dTAB2 Msn is able to activate dTAK1, although only inefficiently. In the wild-type situation, dTAB2 may function as an adaptor that stabilizes such a signaling complex for efficient transduction of the Eiger signal.

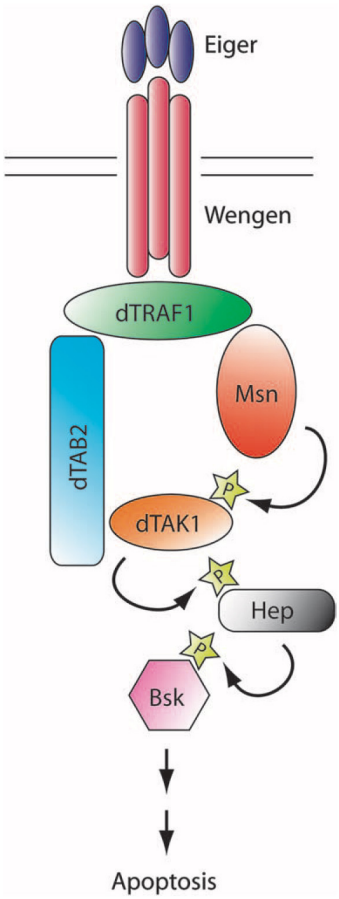

Figure 6.—Proposed model for Eiger signaling in Drosophila. Following the binding of Eiger to Wengen a signaling complex consisting of Msn-dTRAF1-dTAB2-dTAK1 is stabilized, which allows the phosphorylation and activation of dTAK1 by Msn. Subsequently dTAK1 activates the core JNKK-JNK module, consisting of the Drosophila homologs Hep and Bsk.

Even though dTRAF1 exhibited a weaker interaction than dTRAF2 toward dTAB2 and Wengen, we suggest that dTRAF1 rather than dTRAF2 functions as a component of this signaling complex on the basis of the following arguments: (1) Only dTRAF1 but not dTRAF2 binds to Msn (Liu *et al.* 1999); (2) loss-of-function and protein-interaction studies place *dTRAF1* in the JNK pathway and *dTRAF2* in the NF-κB pathway (Liu *et al.* 1999; Shen *et al.* 2001; Cha *et al.* 2003); and (3) males hemizygous mutant for *dTRAF2* do not suppress the Eiger-induced small eye phenotype (not shown; *dTRAF1* alleles are homozygous lethal and could not be properly analyzed—see materials and methods).

In mammalian systems it is not understood how TAK1 is activated. TAB1 is an activator of TAK1, but the

mechanism by which it activates TAK1 and the possible involvement of upstream kinases are not known (Shibuya *et al.* 1996; Sakurai *et al.* 2000). In Drosophila no functional TAB1 homolog has been identified so far. On the basis of our genetic epistasis data, its interaction with dTRAF1, and its homology to MAP4Ks, we propose that Msn functions as an upstream kinase of dTAK1. In mammals NIK and germinal center kinases are structural homologs of Msn. NIK has been demonstrated to act downstream of TAK1 (Ninomiya-Tsuji *et al.* 1999) in NF-κB activation. Several germinal center kinases have been involved in TRAF-mediated activation of JNK (Yuasa *et al.* 1998; Fu *et al.* 1999; Shi *et al.* 1999; Shi and Kehrl 2003). It will be interesting to determine whether one of them plays a role in TNF-induced activation of TAK1. The mapping of other suppressor mutations and the characterization of their corresponding gene products may unravel important aspects of this evolutionarily ancient signaling pathway that has been employed for prominent roles in mammalian development and homeostasis.

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4.6 Identification and Characterization of *Drosophila* Mkk4

Manuscript in the format of a “Genetics Note” in preparation.

A Non-redundant Role for *Drosophila* Mkk4 and Hemipterous in Eiger Signaling

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ABSTRACT

Mutations in *Drosophila Mkk4* strongly suppress Eiger-induced, Jun-N-terminal kinase (JNK)-mediated apoptosis, revealing a non-redundant function for dMkk4 and Hemipterous (dMkk7) in this signaling cascade. These findings are supported by the physical interaction of dMkk4 with the up- and downstream kinases dTAK1 and Basket (dJNK).

The JNK pathway, one of the three major classes of mitogen-activated protein (MAP) kinase pathways (Erk, p38 and JNK), is induced by pro-inflammatory cytokines, such as Tumor Necrosis Factor (TNF) and Interleukin-1 (IL-1), and several forms of environmental stress (e.g. osmotic stress, irradiation, and oxidative stress) (Davis, 2000; Ip and Davis, 1998; Weston and Davis, 2002). In mammals JNK is reported to be activated by the two MAPK kinases (MAPKK) Mkk4 and Mkk7, with Mkk7 mainly involved in TNF- or IL-1-induced JNK activation and both, Mkk4 and Mkk7, required for stress induced activation of JNK (Cuenda, 2000; Holland et al., 1997; Lawler et al., 1997; Moriguchi et al., 1997; Tournier et al., 2001; Tournier et al., 1997). So far only mutations in the *Drosophila* homolog of *Mkk7*, *hemipterous* (*hep*), have been isolated, but not in *Drosophila Mkk4*. Null mutations in *hep* lead to a defect in dorsal closure, a well characterized process in the *Drosophila* embryo that depends on JNK signaling (Glise et al., 1995). This already clearly demonstrates that in this situation Mkk4 cannot substitute for Hep function. Although it has been reported that in mammals Mkk4 and Mkk7 may synergistically activate JNK (Fleming et al., 2000; Lawler et al., 1998), this does not seem to be the case for Hep-mediated Bsk activation in the process of dorsal closure. As already mentioned in mice the situation seems to be more complicated. Both, *Mkk4* and *Mkk7* single mutants are embryonic lethal, but display different phenotypes (Ganiatsas et al., 1998; Nishina et al., 1999; Tournier et al., 2001; Yang et al., 1997), indicating partially overlapping sets of functions.

In a dominant suppressor screen for new components of the Eiger-JNK-pathway in *Drosophila* (Geuking et al., 2005) we identified several mutations in *Drosophila Mkk4*. Some of the EMS-induced mutations suppressing Eiger-induced cell death we obtained in this screen, mapped genetically very close to a deficiency that also suppresses the Eiger-induced small eye phenotype (*Df(3L)Exel6149*). *Df(3L)Exel6149* was identified by screening the whole Exelixis deficiency kit (Parks et al., 2004). Since *Mkk4* is deleted in *Df(3L)Exel614*, we sequenced the coding region of *Mkk4* in those EMS alleles. Indeed molecular lesions were detected in 21 of them (Table 1, Figure 1A). All mutations identified are viable over *Df(3L)Exel6149* or in heteroallelic combinations (but in some cases homozygous lethal due to second hits on the chromosome), confirming that Mkk4 does not crucially participate in dorsal closure of the *Drosophila* embryo.

Allele	DNA Level	Protein Level
G48	G**C**A→G**T**A	Ala10→Lys
G356	653bp ins. at Ser38	AA11 of ins. is a STOP
G673	**C**AG→**T**AG T**T**C→T**A**C	Gln66→STOP Phe184→Tyr
G344	**C**GA→**T**GA	Arg154→STOP
G993	**G**AT→**A**AT	Asp168→Asn
G451	**G**TG→**A**TG	Val171→Met
G201	**G**AT→**A**AT	Asp249→Asn
G343	**G**TG→**A**TG	Val250→Met
G894	G**T**G→G**A**G	Val250→Asn
G136	**C**CG→**T**CG	Pro252→Ser
G863	G**G**T→G**A**T	Gly269→Asp
G414	**C**AG→**T**AG	Gln273→STOP
G39	C**C**G→C**T**G	Pro292→Leu
G583	**C**CG→**T**CG	Pro292→Ser
G657	**G**AT→**A**AT	Asp308→Asn
G504	**G**AG→**A**AG	Glu318→Lys
G270	C**C**C→C**T**C	Pro325→Leu
G587	T**G**G→T**A**G	Trp329→STOP
G1010	5' splice site intron 3: AG/**G**T→AG/**A**T	-
G680	**C**AA→**T**AA	Gln341→STOP
G262	**G**TG→**A**TG	Val361→Met

Table 1 Molecular lesions identified in *Mkk4*. Alleles are ordered according their position in the protein.

Removing one copy of *Mkk4* leads to a potent suppression of the Eiger-induced small eye (Figure 1B, C, and D). Removing two copies of *Mkk4* does not significantly enhance this suppression (Figure 1E). It is important to note that already in hemizygous mutant *hep* males (hypomorphic hep^{1} allele) a very good suppression of the Eiger-induced small eye phenotype is observed (Igaki et al., 2002) (Figure 1F), indicating that in *Drosophila* both MAPKKs, Mkk4 and Mkk7 (Hep), are required for proper transduction of the Eiger signal from dTAK1 to Bsk. This demonstrates that in *Drosophila*, in contrast to mammals, Mkk4 is required for TNF (Eiger)-mediated JNK activation. The effect in hep^{1}–*Mkk4* double mutant males remains to be analyzed. Introducing a *tubulin-Mkk4* rescue transgene reverts the observed dominant suppression indicating that indeed *Mkk4* is responsible for this effect (Figure 1G). However, this rescue transgene seems not to be specific, because it also reverted the suppression brought about by dominant loss of *bsk* (complete) and *dTAB2* (only partially). The same could be observed with a *tub-dTAB2* rescue transgene, that also reverted the suppression observed by dominant loss of *bsk* but not the one brought about by the uncharacterized allele *G56* for example (not shown). This effect can be explained by the fact that the *tub*-promotor expresses the respective gene slightly higher than at endogenous levels, thereby slightly over-activating the pathway but not yet fully enabling the dominant negative effect observed with UAS-constructs. In our case, this results in the observed non-specificity of these rescue

transgenes mainly because *GMR-egr* is a very sensitive background and because dominant, and not recessive, effects are reverted. The slight over-activation induced by use of the *tub*-constructs was observed to only completely revert the dominant suppression induced by loss of a downstream suppressor, offering the possibility to use this effect as an epistasis tool. A genomic rescue construct would most likely be specific, as it is the case for the genomic *dTAK1* rescue construct, which specifically reverts the suppression caused by loss of *dTAK1* (not shown).

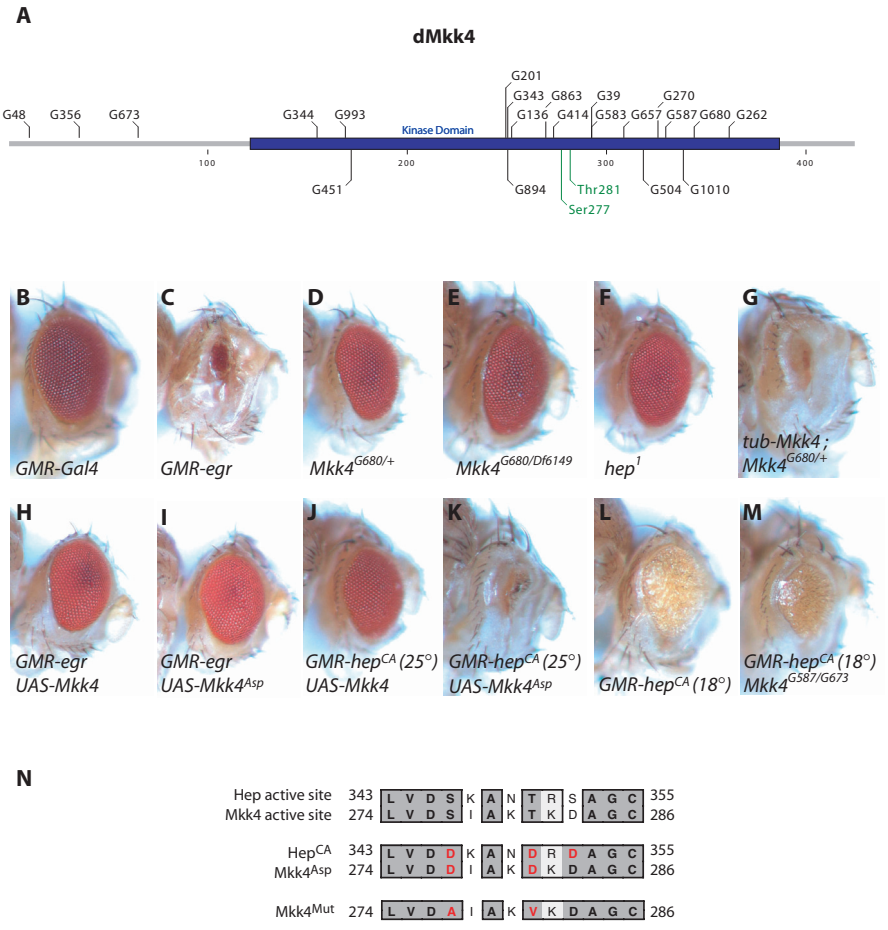

Figure 1 (A) Schematic representation of dMkk4. Alleles (black) and Ser/Thr phosphorylation sites (green) are indicated. (B)-(I) are in a *GMR-egr* (*GMR-Gal4 UAS-egr*) background. (B) *GMR-Gal4/+* control eye. (C) *GMR-egr/+* small eye. (C) *GMR-egr/+; Mkk4^{G680}/+*. Removing one copy of *Mkk4* suppresses the small eye phenotype. (D) *GMR-egr/+; Mkk4$^{G680/Df6149}$*. Removing both copies of *Mkk4* does hardly improve the suppression. (F) *hep^{1}; GMR-egr/+*. Males hemizygous mutant for a hypomorphic *hep* allele display a strong suppression of the small eye. (G) *GMR-egr/tub-Mkk4; Mkk4^{G680}/+*. A *Mkk4* rescue transgene reverts the dominant suppression observed by loss of one copy of *Mkk4*. (H) *GMR-egr/UAS-Mkk4*. Co-expression of Mkk4 has dominant negative effect on Eiger signal transduction. (I) *GMR-egr/UAS-Mkk4^{Asp}*. The same is true for Mkk4^{Asp}. (J) *GMR-hep^{CA}/UAS-Mkk4* (25°). Co-expression of *Mkk4* also suppresses the small eye phenotype induced by Hep^{CA}. (K) *GMR-hep^{CA}/UAS-Mkk4^{Asp}* (25°). Co-expression of *Mkk4^{Asp}* does not suppress the small eye phenotype induced by Hep^{CA}. (L) *GMR-hep^{CA}/+* (18°). Weaker expression of *hep^{CA}* leads to a less severe small eye phenotype. (M) *GMR-hep^{CA}/+; Mkk4$^{G587/G673}$* (18°). This phenotype is not suppressed, even when both copies of *Mkk4* are removed. (N) Indicated are the mutations introduced in Mkk4^{Asp} and Mkk4^{Mut}.

To confirm that Mkk4 indeed acts, like Hep, at the level of a MAPKK, epistasis experiments in flies and cells as well as protein interaction studies were performed. The small eye phenotype induced by expression of hep^{CA} in the *Drosophila* eye (Figure 1L) is not suppressed by removing one (not shown) or both copies of *Mkk4* (Figure 1M) placing Mkk4 upstream or in parallel to Hep. RNAi experiments in S2 cells place Mkk4 downstream of the MAPKKK dTAK1 verifying Mkk4 to function as a classical MAPKK: expression of dTAK1 strongly induces the activity of an AP1-luciferase-reporter in S2 cells (Figure 2A). RNAi against *hep* and *Mkk4* together significantly reduces this activity (Figure 2A). Single RNAi treatment against either of the two kinases was not sufficient to reduce the luciferase signal, which could be explained by the very strong induction obtained by expression of dTAK1 (Figure 2A). In flies, lowering the dosage of either Hep or Mkk4 is sufficient to suppress the Eiger-induced small eye phenotype (Figure 1D, and F). LPS-induced JNK phosphorylation in S2 cells, which is widely used to mimick an innate immune response, is also sensitive to RNAi against either *hep* or *Mkk4* (Figure 2B), confirming previous reports indicating that both, Mkk4 and Hep, are required in *Drosophila* innate immune response (Boutros et al., 2002; Chen et al., 2002). In agreement with this the reduction in phosphorylated JNK levels is enhanced when both kinases are targeted by RNAi at the same time (Figure 2B). This experiment also demonstrates that RNAi against *Mkk4* efficiently knocks down Mkk4 levels.

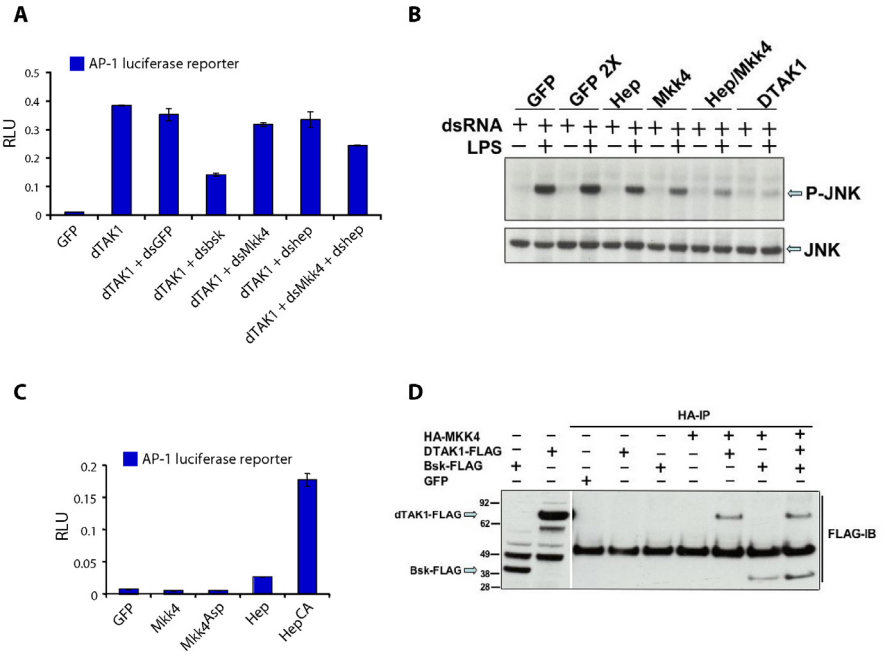

Figure 2 (A) RNAi against *Mkk4* and *hep* together significantly reduces dTAK1-induced AP-1 reporter activity. (B) RNAi against *Mkk4* and/or *hep* reduces phosphorylated JNK levels induced by LPS. (C) In contrast to Hep and Hep^{CA}, Mkk4 and $Mkk4^{Asp}$ do not induce AP-1 reporter activity on their own. (D) Mkk4 physically interacts with dTAK1 and Bsk.

Further evidence suggesting that Mkk4 indeed acts as a MAPKK was obtained from protein interaction studies. Co-immunoprecipitations revealed that Mkk4 physically interacts with its upstream kinase dTAK1 as well as with its downstream kinase Bsk. When expressed in S2 cells, N-terminally HA tagged Mkk4 co-immunoprecipitated both, C-terminally FLAG tagged dTAK1 and Bsk (Figure 2D).

In contrast to the intrinsic activity of Hep^{CA} (strong) and Hep^{WT} (weak), wild type Mkk4 does not activate the JNK pathway when overexpressed in cells (Figure 2C) or in flies (not shown). Interestingly, wild type Mkk4 has a dominant negative effect when co-expressed with Eiger (Figure 1H) or Hep^{CA} (Figure 1J) in flies. This is probably due to its interaction with Bsk and dTAK1. Co-expression of Mkk4 probably titrates away Bsk and dTAK1. In an attempt to generate a constitutive active Mkk4 ($Mkk4^{Asp}$), we introduced the Ser277→Asp and Thr281→Asp mutations, which corresponds to the mutations that were introduced to generate Hep^{CA} (Adachi-Yamada et al., 1999) (Figure 1N). Surprisingly, $Mkk4^{Asp}$ is not constitutive active, either in flies (not shown) or in cells (Figure 2C), but also dominantly suppresses *GMR-egr* (Figure 1I) but not *GMR-hep^{CA}* (Figure 1K). This difference between $Mkk4^{WT}$ and $Mkk4^{Asp}$ could be explained by assuming that $Mkk4^{Asp}$ is still able to bind to dTAK1 but no longer binds to Bsk. Co-IP experiments to support this hypothesis remain to be done. A kinase dead version of Mkk4 ($Mkk4^{Mut}$) where mutations Ser227→Ala and Thr281→Val were introduced (Figure 1N) behaved identical to $Mkk4^{WT}$ (not shown).

In this study we isolated for the first time mutations in *Drosophila Mkk4*. Our genetic and biochemical experiments demonstrate a non-redundant role for Mkk4 as a MAPKK in the Eiger pathway. It seems that in *Drosophila* both MAPKKs, Hep and Mkk4, are required to induce JNK after TNF stimulation. Since Mkk4 mutants are homozygous viable and do not display any obvious phenotype, we tested their behavior under certain stress conditions. We did not observe any obvious effect under oxidative stress (Paraquat). *hep^{1}* hemizygous males are reported to be hypersensitive to paraquat (Wang et al., 2003). Whether this sensitivity is enhanced in *hep^{1}-Mkk4* double mutant males remains to be analyzed. Furthermore wing hairs in Mkk4 mutants do not display any defect in planar cell polarity, a process that is also regulated by JNK signaling (Klein and Mlodzik, 2005). Again *hep^{1}-Mkk4* double mutants remain to be analyzed. Since there is evidence from cell culture experiments that both JNKKs, Hep and Mkk4, are required for *Drosophila* innate immunity (Boutros et al., 2002; Chen et al., 2002), we are currently analyzing whether homozygous *Mkk4* mutants display any innate immunity defect.

4.7 Identification of Lilliputian and Characterization of its Role in Eiger Signaling

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Lilliputian Cooperates with dJun and dFos to Mediate Eiger-Induced Transcription

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ABSTRACT

Here we describe the identification of mutations in *lilliputian (lilli)*, isolated in a screen for suppressors of Eiger-induced apoptosis. Genetic evidence, supported by biochemical interaction studies, suggests that the three transcription factors Lilli, dJun (Jra) and dFos (Kayak) cooperatively mediate Eiger-induced transcription.

In a screen for dominant suppressors of Eiger-induced apoptosis (Geuking et al., 2005) a lethal complementation group, named *L1* (*G127, G377, G453, G500*), was isolated. All four alleles representing *L1* dominantly suppress the Eiger-induced small eye phenotype (Figure 1B, C, and D). Genetic mapping relative to the *GMR-Gal4* insertion revealed that complementation group *L1* is located on the left arm of the second chromosome. In order to further narrow down the region of interest, a high resolution FLP (fragment length polymorphism) mapping effort was employed (Zipperlen et al., 2005). For this purpose we used FLPs present between the *GMR-Gal4* chromosome and the reference chromosomes FRT40 or EP2L. Analysis of recombinants generated between the reference chromosome and the mutated *GMR-Gal4* chromosome revealed that *L1* is located between FLP 2L027 (23B6) and 2L033 (24C7) (Berger et al., 2001) (Figure 1A). In a next step deficiencies of this region were tested for dominant suppression of the Eiger-induced small eye phenotype and complementation of *L1*. Of analyzed deficiencies only *Df(2L)C144* (22F3-4; 23C3-5) suppressed Eiger-induced apoptosis (Figure 1E) and failed to complement all four *L1* alleles, suggesting that the gene affected in *L1* is deleted in this deficiency. Using overlapping deficiencies (*Df(2L)Exel6008, Df(2L)Exel6277*) (Parks et al., 2004) that complemented *L1* and failed to suppress the small eye phenotype (not shown), the candidate region could be narrowed down to the proximal part of *Df(2L)C144*. This is also consistent with the FLP data, which indicated that the mutations lie proximal to 2L027 (Figure 1A). We tested available loss of function alleles of genes in this region applying the same criteria as described above. Interestingly, mutations in *lilliputian* (*lilli*^{4U5}, *lilli*^{15D1}) (Dickson et al., 1996; Wittwer et al., 2001) were able to suppress the small eye phenotype (although weaker than *L1*) and failed to complement *L1* (Figure 1F). To confirm that *L1* represents *lilli*, the *lilli* coding region of the four isolated alleles was sequenced, revealing molecular lesions in three of them (Table 1). Results of epistasis experiments with a constitutive active form of Hep (Hep^{CA}) (Adachi-Yamada et al., 1999) were consistent with a role for *lilli* downstream of *hep*. The small eye phenotype induced by expression of *hep*^{CA} is suppressed by removing one copy of *lilli* (Figure 1G, and H).

Allele	DNA Level	Protein Level
G127	**A**AG→**T**AG	Lys781→STOP
G377	5' splice site intron 6: AG/**G**T→AG/**A**T	-
G453	-	-
G500	**C**AG→**T**AG	Gln861→STOP

Table 1 Molecular lesions identified in *lilli*. *G453* may carry a mutation outside of the coding region.

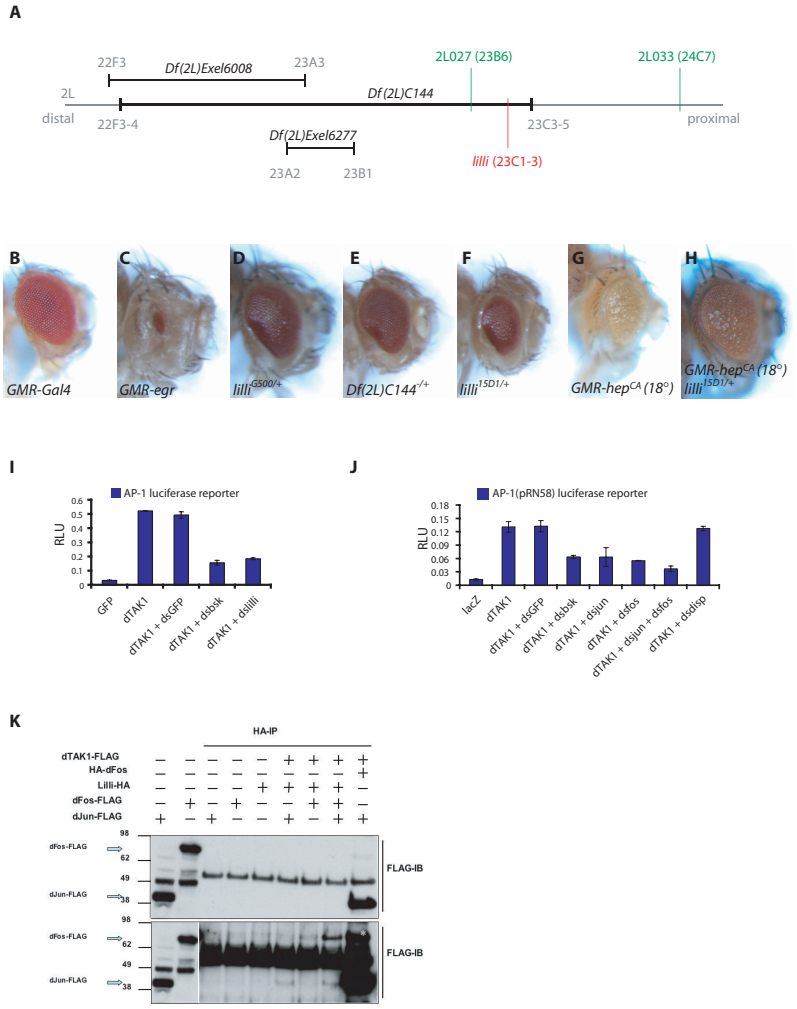

Figure 1 (A) Schematic represntation of the *lilli* locus. (B) *GMR-Gal4/+* control eye. (C) *GMR-egr/+* small eye. (D)-(F) are in a *GMR-egr* (*GMR-Gal4 UAS-egr*) background. (D) *GMR-egr/lilli*^{G500}. Removing one copy of *lilli* suppresses the small eye phenotype. (E) *GMR-egr/Df(2L)C144. Df(2L)C144* dominantly suppresses the small eye phenotype. (F) *GMR-egr/lilli*^{15D1}. A previously identified *lilli* allele also suppresses the small eye although weaker. (G) *GMR-hep*^{CA}*/+* (18°). Expression of *hep*^{CA} also leads to a apoptotic small eye phenotype. (H) *GMR-hep*^{CA}*/lilli*^{15D1} (18°). Removing one copy of *lilli* also suppresses this phenotype. (I) RNAi against *lilli* reduces the dTAK1-induced AP-1 reporter activity. (J) Also RNAi against *djun* or/and *dfos* reduces the activity of an AP-1 (pRN58) luciferase reporter. RNAi against *dispatched (disp)* is used as a negative control. (K) Lilli physically interacts with dJun and dFos. * indicates a non-specific band. Upper panel: short exposure. Lower panel: long exposure.

lilli encodes a large HMG box-containing transcription factor (1673aa) which has already been implicated in several pathways such as the Dpp-, Wg-, Ras-, Raf- and growth regulating pathways as well as in transcriptional regulation of cytoskeletal functions and embryonic segmentation (DasGupta et al., 2005; Dickson et al., 1996; Greaves et al., 1999; Muller et al., 2005; Rebay et al., 2000; Su et al., 2001; Tang et al., 2001a; Wittwer et al., 2001). *lilli* is the single *Drosophila* homolog of the human FMR2/AF4-related gene family (Su et al., 2001; Tang et al., 2001a; Wittwer et al., 2001). Mutations in these genes have been implicated in

human mental retardation and cancer (Chakrabarti and Davies, 1997; Gecz et al., 1997; Gecz et al., 1996; Gu et al., 1992; Gu et al., 1996; Taki et al., 1999).
From mammalian studies it is known that HMG box-containing proteins like Tcf or Lef may interact with other transcription factors such as AP-1 (Jun/Fos) (Nateri et al., 2005; Rivat et al., 2003). As already mentioned in Geuking et al. (2005), *djun* was genetically confirmed to be involved in Eiger signaling. Mosaic flies with eyes homozygous mutant for *djun* display a suppression of the Eiger-induced small eye phenotype (not shown). In addition RNAi against either *djun, dfos* or *lilli* reduces the activity of an AP-1-luciferase-reporter induced by dTAK1 in *Drosophila* cells (Figure 1I, and J). Co-expression of a dominant negative version of dFos in flies also suppresses Eiger-induced apoptosis (not shown). Overexpression of any of them (*djun, dfos, lilli*) does not activate the pathway either in flies (not shown) or in cells. Co-overexpression of combinantions or all three of them also failed to activate the AP1-luciferase-reporter (Narasimamurthy, 2006). To test whether Lilli might cooperate with dJun and dFos in order to transduce the Eiger signal, we tested whether Lilli physically interacts with dJun or dFos. Interestingly, co-immunoprecipitations (IP) revealed a weak physical interaction between Lilli-dJun and Lilli-dFos in dTAK1 stimulated S2 cells. HA tagged Lilli co-immunoprecipitated with FLAG tagged dJun and dFos (Figure 1K). Interestingly the strong interaction between dJun-dFos in stimulated cells is weakened when Lilli is co-expressed, although *lilli* co-overexpression does not have a dominant negative effect on Eiger-induced apoptosis. Whether this interaction is still observed in unstimulated cells remains to be tested.

Since *lilli* was identified in a number of *GMR*-based genetic screens, it remains a possibility, that *lilli* is important for proper transcription from the *GMR* promoter (discussed in Tang et al., 2001a). Based on our RNAi experiments in cells, where *tubulin-Gal4* but not *GMR-Gal4* is used, and the physical interaction we observed between AP-1 and Lilli, we are confident that Lilli's influence on the *GMR* promoter is not the major contribution to the suppression of the small eye phenotype we observe. In addition the small eye phenotype observed when either *UAS-hid* or *UAS-egfr*^{DN} is driven by *GMR-Gal4* is not suppressed (not shown). Therefore we conclude that Lilli is a true component of the Eiger pathway in *Drosophila* that cooperates with dJun and dFos in target gene transcription. It will be interesting to find out whether a Lilli related or any other HMG box-containing transcription factor is also involved in TNF-induced, AP-1-mediated transcription in humans.

Although *lilli* has already been implicated in a number of processes, we provide the first molecular partner for one of its functions. We think that Lilli has very pleiotropic functions and possibly acts as a cofactor for a lot of different transcription factors (discussed in Tang et al., 2001a). Whether its cooperativity with AP-1 is dedicated only to the Eiger pathway or is also important in other AP-1-dependent processes, such as thorax- or dorsal closure, remains to be determined.

4.8 Recombination-Based “Complementation” Analysis

With the exception of the *bsk* and *L1* lethal complementation groups, the thus far identified genes are homozygous viable. Therefore we developed another genetic assay to determine whether two suppressor mutations affect the same or different genes.

Two mutagenized chromosomes carrying independent suppressor mutations were allowed to recombine in females. When crossing these virgins back to *GMR-egr*/*CyO* males, two different scenarios are possible. First, the non-*CyO* progeny contains flies displaying small and suppressed eye phenotypes (Figure 7b). This means that a recombination event was able to bring the two suppressor mutations together on one chromosome, and at the same time generated a non-mutant chromosome. However, if the two independent suppressor mutations affect the same gene, such an event is very unlikely and the non-*CyO* progeny will only consist of flies with a suppressed eye phenotype (Figure 7a).

This assay was applied for two groups of three suppressor mutations located on the third chromosome. One group consisted of *G467*, *G781* and *G870* the other consisted of *G599*, *G602* and *G698*. These were put into the respective group based on their relative genetic distance to RFP4, RFP15 and RFP5 (see Appendix). Recombination-based “complementation” analysis revealed that all six of them affect different genes. In any tested combination, non-*CyO* progeny with small eyes was obtained. Also in this analysis, the relation between the numbers of flies with small or suppressed eye phenotype in theory gives the relative genetic distance between the two independent suppressor mutations. But in our experiments too few progeny was counted to obtain a statistically significant genetic distance.

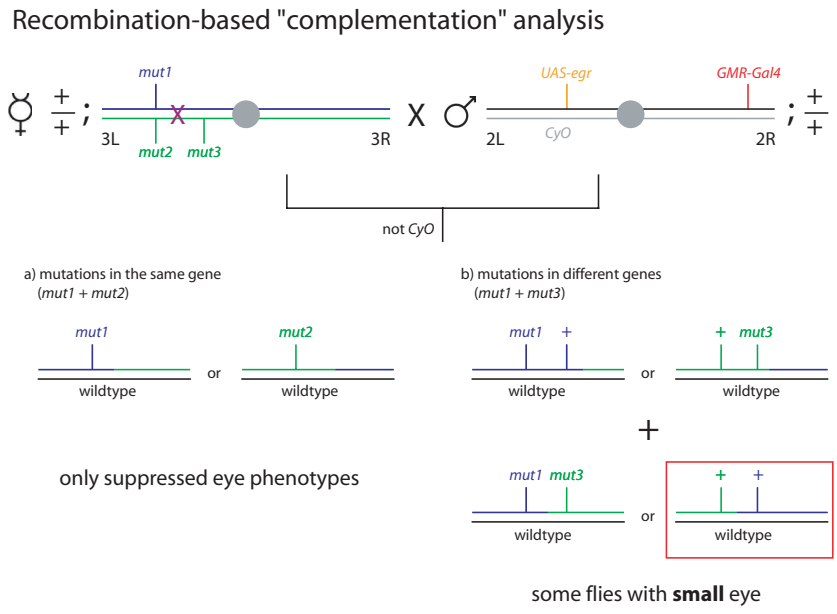

Figure 7 Crossing scheme for “recombination-based complementation analysis”.

4.9 Suppressor *G870* Deletes Chromosomal Region 75C

Genetic rough mapping relative to RFP4, RFP15 and RFP5 revealed that the three suppressor mutations *G467*, *G781* and *G870* roughly map to the region of the known pro-apoptotic genes *hid*, *rpr*, *grim* and *skl* at cytological position 75C (Figure 8). To test whether any of these three suppressor mutations affects a pro-apoptotic gene, a complementation analysis with the lethal deficiency *Df(3L)H99*, which deletes *hid*, *rpr* and *grim* but not *skl* (White et al., 1994) was conducted. *Df(3L)H99* is known to suppress the small eye phenotype (Moreno et al., 2002b). One of the three mutations, *G870*, failed to complement the lethality of *Df(3L)H99*. Subsequently the coding region of the four pro-apoptotic genes was sequenced in the *G870* stock, and also in the *G467* and *G781* stocks because these probably harbor viable mutations in one of the four genes. Since all three chromosomes (*G476*, *G781*, *G870*) are homozygous lethal and therefore balanced with *TM6b*, it is likely to detect polymorphisms in the coding sequence of any of the four genes analyzed. Surprisingly, sequence analysis did not reveal molecular lesions in these genes in any of the stocks. Importantly however, in the case of *G870*, not a single double peak (polymorphism) was detected in any of the sequences analyzed. This lead to the hypothesis that *G870* may carry a deletion at 75C and that in our analysis only pieces of the *TM6b* chromosome were amplified and sequenced. In order to confirm this hypothesis, further complementation tests with *G870* were performed. In addition to *Df(3L)ED224* and *Df(3L)ED225*, which were isolated in the deficiency screen and also delete genes at 75C, *G870* also failed to complement the lethality of the small deficiencies *Df(3L)Exel6133* and *Df(3L)Exel6134*. These two deficiencies delete genes just distal and proximal to the pro-apoptotic genes (Figure 8), consistent with the fact that *Df(3L)Exel6133* and *Df(3L)Exel6134* do not suppress the Eiger-induced small eye phenotype. Based on this complementation results we conclude that *G870* indeed is a deletion that removes not only *hid*, *rpr*, *grim* and *skl* but also more distal and proximal regions. A further fine mapping of the breakpoints of *Df(3L)G870* was not conducted. *Df(3L)G870* represents another validation for the screen setup. The fact that *Df(3L)G870* deletes *hid*, *rpr*, *grim* and *skl* combined with results from the “recombination-based complementation analysis” indicates that *G467* and *G781,* in theory, must lie in a region flanking *Df(3L)G870*.

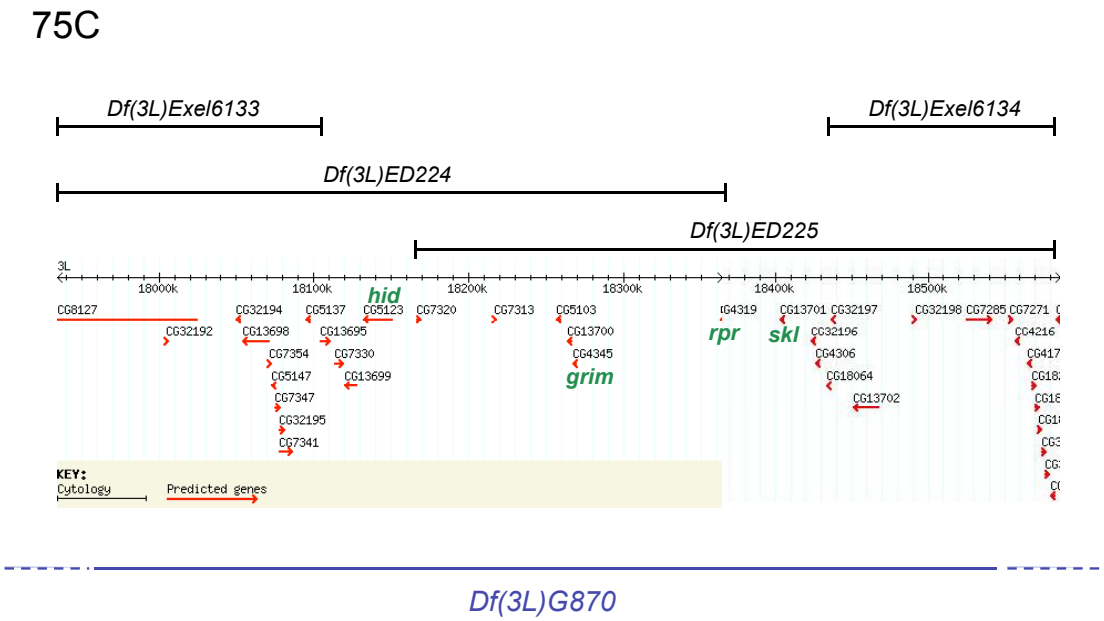

Figure 8 Overview over locus 75C where *hid, rpr, grim and skl* are located.

4.10 *Df(3L)Exel6107*

Df(3L)Exel6107 was isolated in the deficiency screen described earlier, but none of the suppressor mutations isolated from the EMS screen mapped to the same region (64E). As already mentioned, the suppression of the small eye phenotype observed with *Df(3L)Exel6107* could not be confirmed with two independent deficiencies (*Df(3L)ZN47* and *Df(3L)CH20*) that even extend the region deleted in *Df(3L)Exel6107*. This excluded the possibility that the responsible gene may be very small or very weakly accessible by EMS. *Df(3L)Exel6107* was generated by FLP/FRT-mediated recombination between two XP-elements (*P{XP}d08114* and *P{XP}d03136*) which resulted in a w^+-marked XP-element (*P{XP}Exel6107*) inserted exactly at the breakpoint of the deficiency (Parks et al., 2004). The fact that the suppressor present on the deficiency chromosome could not be separated from this w^+ marker (not shown) indicated that locus 64E is responsible for the observed suppression. Since XP elements contain UAS sites (Parks et al., 2004), we thought that the observed suppression of the small eye is not due to a deleted gene but may be due to a gene proximal or distal to the deficiency that is co-expressed when crossed to the *GMR-egr* stock (*GMR-Gal4 UAS-egr*). To test this hypothesis we thought to analyze the potential to suppress the small eye phenotype of the two XP-insertions that were used to generate *Df(3L)Exel6107*. Unfortunately *P{XP}d08114* and *P{XP}d03136* were not available as stocks. However, we could test two other XP-insertions, *P{XP}d07920* and *P{XP}d03195*, inserted in the same intergenic region as *P{XP}d08114* and *P{XP}d03136*, respectively. *P{XP}d07920* is inserted 710bp upstream of *P{XP}d08114* and *P{XP}d03195* is inserted only 37bp upstream of *P{XP}d03136*. Interestingly, *P{XP}d03195*, but not *P{XP}d07920*, also suppressed the Eiger-

induced small eye phenotype. From this we concluded that indeed the UAS sites present in *P{XP}d03195* and *P{XP}Exel6107* are probably responsible for the observed suppression. Since *P{XP}d07920* did not suppress the small eye, we concluded that the responsible gene influenced by the UAS sites should lie proximal to *P{XP}d03195*.

CG13288 is the first gene located downstream (+5kb) of *P{XP}d03195*, that is in the right orientation (Figure 9). *CG13288* encodes an uncharacterized protein containing four predicted transmembrane helices and no other known domains. Whether this gene is indeed responsible for the suppression remains to be confirmed by expression of a *UAS-CG13288* transgene. Since this gene is already quite far away from the UAS sites, another possibility is that an unpredicted gene closer to *P{XP}d03195* is responsible for the observed suppression.

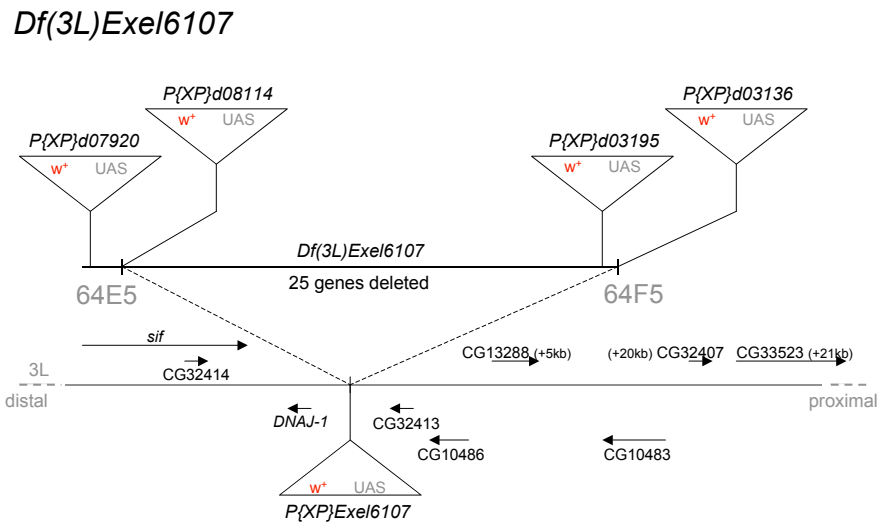

Figure 9 Overview over XP-insertions and *Df(3L)Exel6107* located at 64E-F.

4.11 Overview Chromosome II + III

Figure 10 gives an overview about the chromosomal locations of the Eiger suppressors identified in this study. Grey indicates either genetic markers used for mapping or genes involved in the pathway that were not isolated from the screen, due to reasons discussed earlier. Cs II: *lilli* (green), *bsk* (orange), *dTAB2* (blue), *GMR-Gal4* insertion (red), single hits (black). Cs III: *Mkk4* (blue); Single hits, *Df(3L)Exel6107* and locus 75C in black.

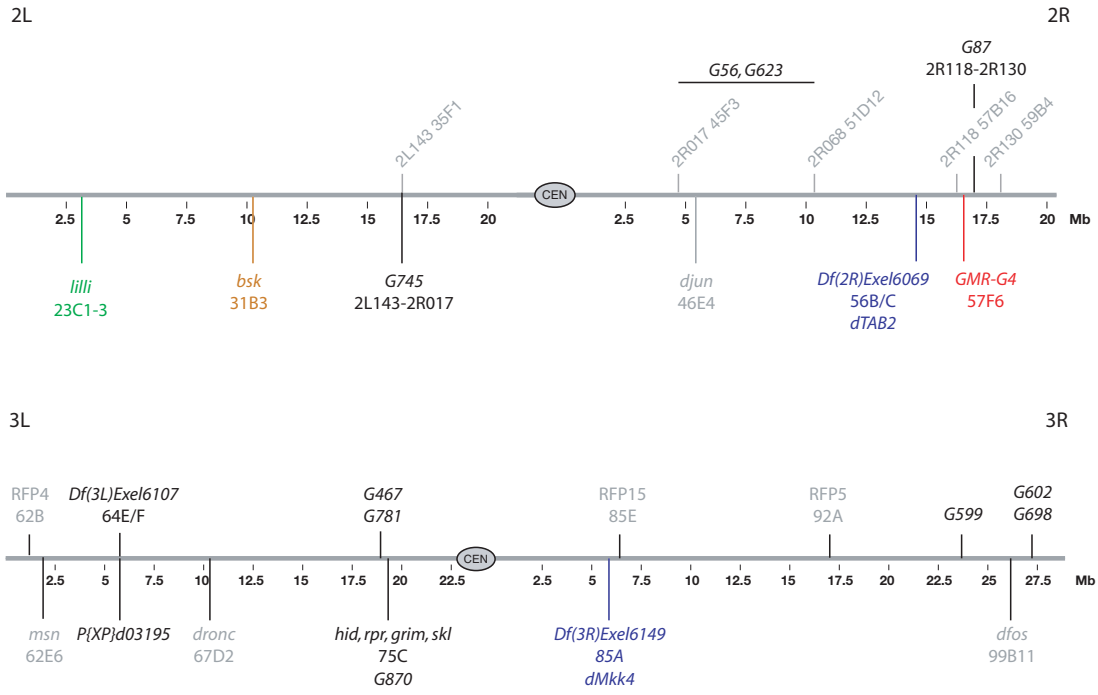

Figure 10 Overview chromosome 2 and 3

5 Discussion

5.1 The EMS Screen

With a very simple genetic setup a very powerful screen could be performed. In addition to genes already known to be involved in the Eiger pathway, such as *bsk*, *dTAK1* and *hid*, we identified mutations in three genes that had not yet been implicated in *Drosophila* Eiger signaling (*dTAB2, Mkk4, lilli*). Mutations in *lilli* have already been identified in other screens, but no mutations affecting *dTAB2* or *Mkk4* were available to date. The high number of alleles obtained in *bsk*, *dTAB2*, *Mkk4* and also in the *GMR-Gal4* transgene suggests that the screen has reached complete saturation, at least for the second and third chromosome. However, it is striking that for the largest gene identified, *lilli* (~5kb CDS), we only obtained four new alleles. From a comparison of the molecular nature of the different *lilli* EMS alleles that were tested (*4U5, 15D1, G127, G377, G453, G500*), there is no reason to assume that the four *lilli* alleles identified in our screen are somehow stronger or have even a dominant negative nature, although $lilli^{4U5}$ and $lilli^{15D1}$ had a weaker potential to suppress the Eiger-induced small eye phenotype than *L1*. Observations by Wittwer et al. (2001) even classify $lilli^{4U5}$ as a strong null allele. One explanation for the low number of alleles obtained is, that the *lilli* locus is weakly accessible for EMS. To confirm this hypothesis the number of flies screened, the number of *lilli* alleles identified and the EMS concentrations used in the different screens in which *lilli* mutants were found, could be compared.

Complete saturation would also mean, that in theory we should also have identified mutations in *hid*, *rpr*, *grim* and *skl*, or at least in one of them. *rpr*, *grim* and *skl* have a very short CDS (180-400bp) and therefore are rarely hit by EMS. The *hid* CDS, however, has approximately the same size as the *Mkk4* CDS (~1.2kb). It is therefore surprising that we identified 21 mutations in *Mkk4*, but isolated only one deletion uncovering the four pro-apopototic genes. One reason for this could be the redundancy between these genes, meaning that removing one copy of just one of the four pro-apoptotic genes in this region was not sufficient to get a suppression that was above the phenotypic threshold level set in the screen.

Most of the suppressor mutations identified in the EMS screen could be assigned to a single gene. There are only eight “single hits” left, which remain to be mapped (see Figure 10 and Appendix). Following a FLP/SNP mapping approach (Berger et al., 2001; Martin et al., 2001; Nairz et al., 2002; Zipperlen et al., 2005) it should be possible to map these suppressors in a straight forward fashion. The crossing scheme for these approaches would be comparable to the one applied for FLP mapping in this work. The identification of these genes may uncover very interesting new components and signaling mechanisms of TNF signal transduction in general.

5.2 The Deficiency Screen

The deficiencies identified in the deficiency screen turned out to be extremely valuable as tools to map the suppressor mutations obtained from the EMS screen. A major advantage of the screened deficiencies is that they are molecularly precisely mapped and in an isogenic background. Another very useful feature, especially of the Exelixis deficiencies, is that they are rather small. The three Exelixis deficiencies identified in our screen delete only 20-27 genes, which dramatically reduced the number of candidate genes in each case. Using overlapping deficiencies can also be helpful in order to further narrow down the region of interest. Some of the Exelixis deficiencies were generated with elements containing UAS sites (XP- and WH-elements) (Parks et al., 2004). In some cases these UAS sites are still present after the recombination event. Depending on the genetic background in which a screen is performed, these UAS sites can have an influence on the phenotype. It is very important to be aware of this fact when using these deficiencies. The elements used to generate the DrosDel deficiencies never contain UAS sites (RS3- and RS5-elements) (Ryder et al., 2004).

For all the deficiencies identified in our screen the responsible gene or UAS-containing element could be identified, proving that such a deficiency screen indeed can be very powerful when combined with other approaches, like an EMS screen.

5.3 Final Model

Table 2 summarizes again all the deficiencies and genes that were identified in the process of this work. Our current view of how the Eiger signal is transduced from the cell membrane to the nucleus is depicted in Figure 11.

Deficiency	**Gene**	**# of Alleles**
-	*dTAK1*	1
-	*bsk*	10
Df(3L)ED224 *Df(3L)ED225*	*hid, rpr,* *grim, skl*	1 (*Df(3L)G870*)
Df(2R)Exel6069	*dTAB2*	39
Df(3R)Exel6149 *Df(3R)ED5296*	*Mkk4*	21
Df(2L)C144	*lilli*	4
Df(3L)Exel6107	*CG13288?**	1
-	*GMR-Gal4*	21 (+5)

Table 2 Summary of deficiencies and genes identified in this work.
* not yet confirmed

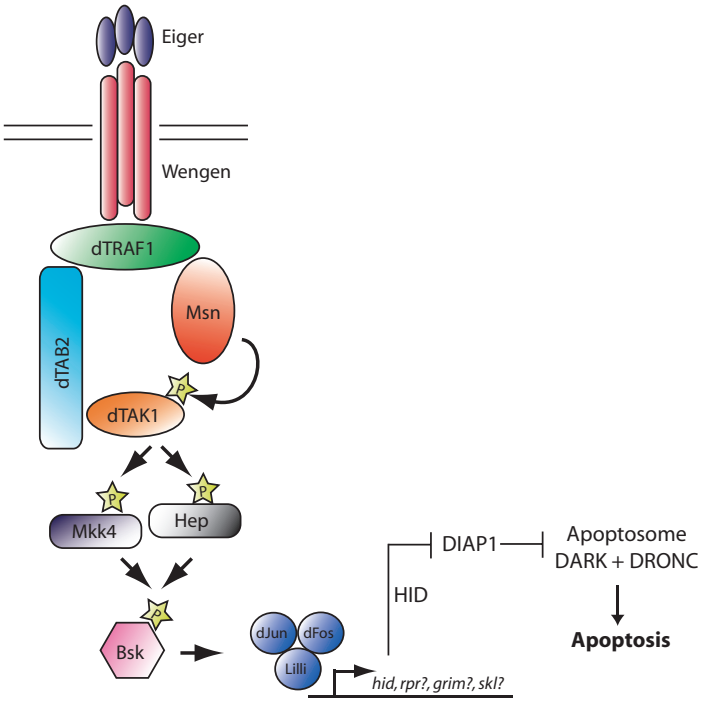

Figure 11 Current view of transduction of the Eiger signal from the membrane to the nucleus.

Mammalian MAPKKKs other than TAK1 (ASK1, MLK and MEKK1) have been implicated in TNF-induced JNK activation as well (Chang and Karin, 2001). An observation that can be explained by the number of different receptors existing in mammals: some of them might use a different mechanism to induce JNK signaling. In mammals the TAK1-TAB2 module was first implicated in Toll-induced JNK and NFκB activation, but then also shown to be involved in TNF-mediated induction of these pathways (Ishitani et al., 2003; Morlon et al., 2005; Takaesu et al., 2000; Takaesu et al., 2001). So far there is no evidence that Eiger also induces NFκB, although predicted since it acts through the TAK1-TAB2 module, which is an activator of NFκB. The fact that the TAK1-TAB2 module is indeed required for the IMD (TLR) pathway in *Drosophila* (D. Ferrandon, unpublished) and that Eiger solely acts through this module, indicates that this is the primordial mechanism of TLR/TNF-induced JNK activation and that other ways to induce TNF-mediated JNK activation probably arose later in evolution. In addition it also seems that signaling mechanisms evolved together with Toll signaling, such as the FADD-Caspase-8 module, may have been adopted by TNF signaling at later stages of evolution (Moreno et al., 2002b).

It is an interesting observation that, in contrast to mammals, in *Drosophila* Mkk4 seems to be strictly required for TNF-induced JNK activation. Elucidating other roles of Mkk4, not detected in our experiments, by further characterization of the *Mkk4* null alleles isolated in this work will be an important step in finding the reasons for this difference. In addition it will be exciting to elucidate whether TNF-induced mammalian AP-1 also requires co-factors similar to Lilli in *Drosophila*.

5.4 Physiological Functions of Eiger

A very important question remains: what are the funcions of TNF/Eiger in *Drosophila*? Indeed very little is known about the physiological functions of *egr*. Flies homozygous mutant for *egr* are viable and display no obvious phenotype (Igaki et al., 2002). Igaki et al. reported that the observed JNK activity behind the morphogenetic furrow in *Drosophila* eye imaginal discs is reduced in *egr* mutants (Igaki et al., 2002). However, this does not seem to have any morphological or developmental consequences. Hence *egr* mutants have either a phenotype that is too subtle to detect, or display a phenotype only under certain conditions. The only condition reported so far where *egr* mutants display a phenotype is upon Salmonella infection: flies homozygous mutant for *egr* have a delay in Salmonella-induced lethality (Brandt et al., 2004). It is thought that Salmonella infection leads to a condition comparable to the TNF-inducible metabolic collapse in vertebrates (Brandt et al., 2004), as a consequence of this the flies die. Whether the observed delay in lethality is due to the lack of Eiger-induced JNK activation remains to be confirmed by testing the effect in *dTAK1*, *dTAB2* or *Mkk4* mutants. Under other stress conditions such as bacterial or fungal infection, oxidative stress or X-ray, *egr* mutants displayed no phenotype different from wildtype flies (see Appendix). Also, generating a situation where *egr* mutant cells are confronted with heterozygous cells in the *Drosophila* eye did not reveal any difference between mutant and control heterozygous cells (not shown). In a process called “cell competition”, cells that proliferate faster than neighboring cells actively kill the slower growing cells by activating JNK-mediated apoptosis in these cells (Moreno et al., 2002a). Eiger would be the perfect signal sent from the faster growing cells to the slower growing ones. Clones overexpressing *brinker (brk)* are eliminated from the center of the wing imaginal disc by cell competition (Moreno et al., 2002a). In such clones a clear upregulation of Eiger was detected by antibody staining (Moreno E., unpublished). Whether this upregulation can also be observed at the transcriptional level can easily be addressed by using the *egr-lacZ* lines described in the Appendix. However, since Eiger was observed to be upregulated in the dying cells and not in the surrounding cells, it can’t be the signal that is sent out by the faster growing cells. One hypothesis is, that Eiger acts in an autocrine fashion and mainly kills the cells that express *egr*. This is consistent with the observation that clones overexpressing *eiger* only kill themselves and one row of surrounding cells (Moreo E., unpublished), unlike a clone overexpressing a soluble form of Eiger that can travel further (Narasimamurthy, 2006). This also indicates that under overexpressing conditions Eiger is not always cleaved and remains on the membrane, explaining its proposed partial autocrine action. This scenario implies that there must exist a signal coming from the faster growing cells that induces Eiger in the slower growing cells. However, in an *egr* mutant background *brk* overexpressing clones still died although slightly slower. This could be explained by redundancy at the level of the inducer. It would be interesting to test whether *brk* overexpressing clones behave differently in a *dTAK1*, *dTAB2* or *Mkk4* homozygous mutant background.

The elucidation of the primordial function of TNF/Eiger in *Drosophila* will be a very interesting task. The fact that lower organisms, such as *C. elegans*, do not encode a TNF homolog in their genome makes it even more interesting to decipher the origins of the evolution of TNF.

5.5 Outlook

The identification of mutations in *dTAB2* and *Mkk4*, for the first time, will allow us to investigate the role of these ptoreins not only in Eiger signaling but, importantly, also in other processes. The availability of these mutants will therefore be of high interest. Our main interest is to decipher molecular mechanisms of TNF/Eiger signaling. An important next step to achieve this goal will be to map the genes that are affected in the still uncharacterized suppressor mutations (“single hits”) found in our screen. Possible other approaches to identify new components or regulators of this pathway are: (1) an *in vivo* RNAi screen or (2) an *in vivo* overexpression screen. For both screens the same genetic tool that was useful in this work (*GMR-Gal4 UAS-egr*), could be applied. A collection of stocks, which will be available soon (B. Dickson, unpublished) carrying UAS-hairpin constructs of all the predicted *Drosophila* genes can easily be screened for suppressors of the small eye phenotype when crossed against the *GMR-egr* stock. In order to identify negative components or regulators a similar collection containing defined comparable UAS-insertions of every predicted gene could be screened (J. Bischof, unpublished). Since the small eye phenotype is not suppressed in *wgn* homozygous mutant flies, the two screens described in this work and the RNAi screen mentioned above could be performed in a *wgn* homozygous mutant background. This strategy would allow us to overcome the problem of possible redundancy at the level of the receptor. The redundancy is suggested by the fact that the Eiger-induced small eye phenotype is not suppressed in *wgn* homozygous mutant flies. Since it would be quite labour intensive to filter out all the known components that would again be hit in such an EMS screen, the most promising approach would be the above mentioned RNAi screen in a *wgn* homozygous mutant background. In this way, every predicted gene could be tested and depending on the efficiency of the RNAi line components that need more than 50% reduction to suppress the small eye phenotype could also be identified. The identification and characterization of further novel components or regulators may uncover important aspects of this evolutionarily ancient signaling pathway that plays important roles in mammalian development and disease.

6 Appendix

6.1 EMS Alleles - 2nd Chromosome

Allele	v/l	Suppression	Suppr. of *GMR-hep*^{CA}	Affected gene, mutation, comments	%rgd to *GMR-Gal4*	FLP data
G56 (*)	l	very good	no	?, no mutation in dTAB2 CDS	60(215/361)*	2R017
G58 (*)	l	very good	dTAB2^{G71} no	dTAB2 Gln119 -> STOP	21 (29/137)*	2R068?/109
G71 (*)	l	very good	no	dTAB2 Gln40 -> STOP	18 (37/120)*	2R096/109
G87 (*)	l	middle	no	?, no mutation in dTAB2 CDS	9 (22/241)*	2R118/130
G127 (*)	l	very good	lilli^{15D1,4U5} yes	lilli Lys781 -> Stop	69 (62/92)*	2L
G147 (*)	l	very good	-	no mutation in dTAB2 CDS	3 (6/190)*	Inversion?
G179 (*)	l	middle	bsk^{G258} yes	bsk Ser127 -> Phe	124(105/85)*	2L
G211 (*)	l	very good	dTAB2^{G71} no	dTAB2 C769 -> G, N770 -> C	12 (24/193)*	2R096/109
G227 (*)	v	very good	dTAB2^{G71} no	dTAB2 Gln713 -> STOP	14 (34/240)*	2R109/83?
G245 (*)	v	very good	dTAB2^{G71} no	dTAB2 7bp deletion	23 (45/199)*	-
G253 (*)	l	very good	dTAB2^{G71} no	dTAB2 Gln544 -> STOP	19 (49/252)*	2R109/83?
G258 (*)	l	good	yes	bsk Trp232 -> STOP	84 (91/108)*	2L
G271 (*)	l	very good	dTAB2^{G71} no	dTAB2 Gln728 -> STOP	19 (51/259)*	2R109/83?
G275 (*)	l	very good	dTAB2^{G71} no	dTAB2 Gln581 -> STOP	19 (36/193)*	2R068?/109
G291	l	total	-	GMR-Gal4	0 (0/810)	-
G303	v	good	-	GMR-Gal4, Inr not rescued	0 (0/1050)	-
G497	l	good	-	GMR-Gal4	0 (0/212)	-
G532	l	total	-	GMR-Gal4	0 (0/153)	-
G560	l	total	-	GMR-Gal4	0 (0/128)	-
G562	l	total	-	GMR-Gal4	0 (0/211)	-
G578	l	total	-	GMR-Gal4	0 (0/170)	-
G581	l	total	-	GMR-Gal4	0 (0/201)	-
G638	l	good	-	GMR-Gal4, Inr not rescued	0 (0/85)	-
G639	l	middle	-	GMR-Gal4, Inr not rescued	0 (0/87)	-
G672	l	total	-	GMR-Gal4	0 (0/338)	-
G686	l	good	-	GMR-Gal4	0 (0/212)	-
G689	l	total	-	GMR-Gal4	0 (0/462)	-
G713	l	good	-	GMR-Gal4	0	-
G769	l	total	-	GMR-Gal4	0	-
G817	l	total	-	GMR-Gal4	0	-
G840	l	good	-	GMR-Gal4, Inr not rescued	0	-
G847	l	very good	-	GMR-Gal4	0	-
G859	l	total	-	GMR-Gal4	0 (0/603)	-
G882	l	total	-	GMR-Gal4	0	-
G918	v	total	-	GMR-Gal4	0	-
G930	v	total	-	GMR-Gal4	0	-
G943	v	total	-	GMR-Gal4	0	-
G998	l	total	-	GMR-Gal4	0	-
G1007	v	good	-	GMR-Gal4, Inr not rescued	0	-
G1019	l	good	-	GMR-Gal4	0	-
G290	l	good	bsk^{G258} yes	bsk Ser208 -> Phe	25 (77/311)	-
G368	l	good	bsk^{G258} yes	bsk Gln115 -> STOP	33 (82/246)	-
G400	l	good	bsk^{G258} yes	bsk Thr181 -> Ile (TPY)	31 (70/225)	-
G418	l	good	bsk^{G258} yes	bsk 3' end intron 3 AG -> AA	31 (112/362)	-
G536	l	middle	bsk^{G258} yes	bsk Ser127 -> Phe	-	-
G577	l	middle	bsk^{G258} yes	bsk Ala143 -> Val	-	-
G688	l	middle	bsk^{G258} yes	bsk Arg308 -> Pro	-	-
G760	l	middle	bsk^{G258} yes	bsk Pro182 -> Ser (TPY)	32 (97/302)	-
G635	v	total	dTAB2^{G71} no	dTAB2 Arg209 -> STOP	2 (8/388)	2R096/109
G715	v	very good	dTAB2^{G71} no	dTAB2 Trp767 -> STOP	2.1 (8/382)	-
G489	l	very good	dTAB2^{G71} no	dTAB2 Gln634 -> STOP	2.3 (22/948)	-
G788	l	good	dTAB2^{G71} no	dTAB2 Gln542 -> STOP	3.6 (13/362)	-
G666	l	good	dTAB2^{G71} no	dTAB2 Gln458 -> STOP	3.7 (16/431)	-
G539	l	good	dTAB2^{G71} no	dTAB2 284bp deletion	3.8 (38/996)	-
G973	l	middle	dTAB2^{G71} no	dTAB2 Gln107 -> STOP	3.8 (17/447)	-
G734	l	good	dTAB2^{G71} no	dTAB2 Gln486 -> STOP	3.9 (22/557)	-
G585	l	good	dTAB2^{G71} no	dTAB2 Gln317 -> STOP	4 (12/306)	2R051/109
G642	l	good	dTAB2^{G71} no	dTAB2 In.2 GT->GA,P450->S	4 (14/358)	-
G856	l	middle	-	no mut. in TAB2, lethal/G790	4 (16/406)	-
G421	l	good	dTAB2^{G71} no	dTAB2 Gln224 -> STOP	4.1 (41/995)	2R068/109
G548	l	good	dTAB2^{G71} no	dTAB2 Gln641 -> STOP	4.1 (38/921)	-
G503	l	good	dTAB2^{G71} no	dTAB2 Gln317 -> STOP	4.2 (35/837)	-
G683	l	good	dTAB2^{G71} no	dTAB2 Gln330 -> STOP	4.3 (12/282)	-
G954	v	good	dTAB2^{G71} no	dTAB2 Gln689 -> STOP	4.4 (19/428)	-

Allele	v/l	Suppression	Suppr. of *GMR-hep*^{CA}	Affected gene, mutation, comments	%rgd to *GMR-Gal4*	FLP data
G825	l	good	dTAB2^{G71} no	dTAB2 53bp deletion	4.5 (23/507)	-
G701	l	good	dTAB2^{G71} no	dTAB2 Trp767 -> STOP	4.6 (20/434)	-
G603	l	good	dTAB2^{G71} no	dTAB2 Gln744 -> STOP	4.8 (26/542)	-
G812	l	good	dTAB2^{G71} no	dTAB2 Glnt20 -> STOP	4.9 (18/369)	-
G576	l	good	dTAB2^{G71} no	dTAB2 Gln66 -> STOP	5.3 (33/616)	-
G545	v	good	dTAB2^{G71} no	dTAB2 Gln712 -> STOP	5.5 (55/998)	-
G520	v	good	dTAB2^{G71} no	dTAB2 Trp767 -> STOP	6 (26/435)	-
G552	l	good	dTAB2^{G71} no	dTAB2 Gln486 -> STOP	6 (18/304)	-
G746	l	very good	dTAB2^{G71} no	dTAB2 Gln633 -> STOP	6 (24/403)	-
G933	l	good	dTAB2^{G71} no	dTAB2 3'end intron 3 AG->AA	6 (16/264)	-
G440	l	very good	-	no mutation in dTAB2 CDS	6.2 (46/742)	2R083/130
G824	l	good	dTAB2^{G71} no	dTAB2 Trp767 -> STOP	6.3 (51/807)	-
G829	l	good	dTAB2^{G71} no	dTAB2 Gln731 -> STOP	6.6 (37/555)	-
G981	v	middle	dTAB2^{G71} no	dTAB2 L570 -> Q, D592 -> N	6.7 (26/388)	-
G790	l	middle	-	no mut. in TAB2, lethal/G856	6.9 (22/320)	-
G928	v	good	dTAB2^{G71} no	dTAB2 Gly794 -> Asp	6.9 (33/481)	-
G867	l	good	dTAB2^{G71} no	dTAB2 Arg233 -> STOP	7 (19/275)	-
G609	l	good	dTAB2^{G71} no	dTAB2 Gln216 -> STOP	7.6 (27/352)	-
G623	l	middle	-	?, dTAB2 Gln779 -> His ??	13 (50/388)	2R017/68
G745	l	good	-	?	28 (76/273)	2L143
G377	l	good	lilli^{15D1,4U5} yes	lilli 5' end intron 6 GT -> AT	29 (165/566)	2L027/33
G500	l	middle	lilli^{15D1,4U5} yes	no mutation in lilli CDS	30 (60/197)	2L027/33
G453	l	middle	lilli^{15D1,4U5} yes	lilli Gln861 -> STOP	36 (44/122)	2L027/33

v = stock homozygous viable
l = stock homozygous lethal
rgd = relative genetic distance
(*) = wt chromosome was mutated
* = rough mapping crossing scheme b)

6.2 EMS Alleles - 3rd Chromosome

Allele	v/l	Suppression	Suppr. of *GMR-hep*^{CA}	Affected gene, mut., comments	%rgd to *RFP4*	%rgd to *RFP15*	%rgd to *RFP5*
G39	l	good	-	Mkk4 Pro292 -> Leu	51 (37/72)	0.6 (1/163)	-
G48	l	good	-	Mkk4 Ala10 -> Val	36 (22/60)	3.1 (5/160)	-
G136	l	good	-	Mkk4 Pro252 -> Ser	44 (69/157)	0.8 (1/125)	-
G201	l	good	-	Mkk4 Pro249 -> Asn	52 (47/91)	1.0 (1/97)	-
G262	l	very good	-	Mkk4 Val361 -> Met	39 (60/154)	0.8 (2/223)	-
G270	l	very good	-	Mkk4 Pro325 -> Leu	39 (15/38)	0 (0/185)	-
G343	l	good	-	Mkk4 Val250 -> Met	44 (58/132)	2.7 (3/110)	-
G344	l	good	no	Mkk4 Arg154 -> STOP	47 (24/51)	0.6 (1/173)	-
G356	l	good	-	Mkk4 653bp ins. at Ser38	52 (62/120)	2.1 (3/142)	-
G414	v	good	no	Mkk4 Gln273 -> STOP	45 (65/143)	2.4 (4/167)	-
G451	l	middle	-	Mkk4 Val171 -> Met	47 (73/154)	1.8 (3/168)	-
G467	l	WEAK* Lost?	yes?	very close to G870!?!	58(123/212)	11(26/230)	28(58/206)
G504	l	weak	-	Mkk4 Glu318 -> Lys	46 (56/122)	2.6 (5/192)	-
G583	l	middle	-	Mkk4 Pro292 -> Ser	50 (68/135)	2.0 (3/153)	-
G587	v	middle	no	Mkk4 Trp329 -> STOP	-	0.5 (1/212)	-
G599	l	middle	yes	TACE 99D1?	53(103/194)	43(68/156)	25(20/80)
G602	l	good	yes	TACE 99D1?	44(64/147)	68(24/35)	46(62/133)
G653	l	middle	-	no mutation in Mkk4 CDS	(21/48)	(1/32)	-
G657	l	weak	-	Mkk4 Asp308 -> Asn	48 (29/62)	0.9 (2/212)	-
G673	l	middle	no	Mkk4 Q66->ST. ,F184->T	47 (74/156)	1.5 (3/194)	-
G680	l	middle	-	Mkk4 Gln341 -> STOP	42 (78/187)	1.0 (2/194)	-
G698	l	WEAK* Lost?	no	TACE 99D1?	45(112/249)	61(74/121)	42(50/118)
G781	l	good	yes? weak	?, no mut in Mkk4 CDS	38 (69/181)	14(16/118)	10
G863	l	middle	-	Mkk4 Gly269 -> Asp	48 (83/173)	0.4 (1/223)	-
G870	l	good	yes	deletion at 75C	-	19 (14/72)	25
G894	l	middle	-	Mkk4 Val250 -> Asn	48 (63/131)	1.4 (3/217)	-
G896	l	weak	-	Mkk4? no PCR products	-	1.1 (1/92)	-
G993	l	good	-	Mkk4 Asp168 -> Asn	46 (68/149)	0.5 (1/193)	-
G1010	l	middle	-	Mkk4 5'end intr.3 GT->AT	46 (96/210)	1.6 (3/182)	-

v = stock homozygous viable
l = stock homozygous lethal
rgd = relative genetic distance
* = because of the very weak suppression, "rec.-based compl. analysis" and "rough mapping" is not very reliable!

6.3 Tested Exelixis Deficiencies

BL# Deficiency	S	BL# Deficiency	S	BL# Deficiency	S	B L# Deficiency	S
7488 Df(2L)Exel6001	-	7553 Df(2R)Exel6071	-	7624 Df(3R)Exel6145	w	7689 Df(3R)Exel6211	-
7489 Df(2L)Exel6002	-	7554 Df(2R)Exel6072	-	7625 Df(3R)Exel6146	-	7690 Df(3R)Exel6212	-
7490 Df(2L)Exel6003	-	7557 Df(2R)Exel6077	-	7626 Df(3R)Exel6147	-	7691 Df(3R)Exel6213	-
7491 Df(2L)Exel6004	-	7558 Df(2R)Exel6078	-	7627 Df(3R)Exel6148	-	7692 Df(3R)Exel6214	-
7492 Df(2L)Exel6005	-	7559 Df(2R)Exel6079	-	7628 Df(3R)Exel6149	s	7693 Df(3R)Exel6215	-
7493 Df(2L)Exel6007	-	7561 Df(2R)Exel6082	-	7629 Df(3R)Exel6150	-	7694 Df(3R)Exel6216	-
7494 Df(2L)Exel6008	-	7562 Df(3L)Exel6083	-	7630 Df(3R)Exel6151	-	7695 Df(3R)Exel6217	-
7495 Df(2L)Exel6009	-	7563 Df(3L)Exel6084	-	7631 Df(3R)Exel6152	-	7696 Df(3R)Exel6218	-
7496 Df(2L)Exel6010	-	7564 Df(3L)Exel6085	-	7632 Df(3R)Exel6153	-	7697 Df(3R)Exel6219	-
7497 Df(2L)Exel6011	-	7565 Df(3L)Exel6086	-	7633 Df(3R)Exel6154	-	7699 Df(1)Exel6221	-
7498 Df(2L)Exel6012	-	7566 Df(3L)Exel6087	-	7634 Df(3R)Exel6155	-	7700 Df(1)Exel6223	-
7499 Df(2L)Exel6013	-	7567 Df(3L)Exel6088	-	7635 Df(3R)Exel6156	-	7702 Df(1)Exel6225	-
7500 Df(2L)Exel6014	-	7568 Df(3L)Exel6089	-	7636 Df(3R)Exel6157	-	7703 Df(1)Exel6226	-
7501 Df(2L)Exel6015	-	7569 Df(3L)Exel6090	-	7637 Df(3R)Exel6158	-	7704 Df(1)Exel6227	-
7502 Df(2L)Exel6016	-	7570 Df(3L)Exel6091	-	7638 Df(3R)Exel6159	-	7705 Df(1)Exel6230	-
7503 Df(2L)Exel6017	-	7571 Df(3L)Exel6092	-	7639 Df(3R)Exel6160	-	7706 Df(1)Exel6231	-
7504 Df(2L)Exel6018	-	7572 Df(3L)Exel6093	-	7640 Df(3R)Exel6161	-	7707 Df(1)Exel6233	-
7505 Df(2L)Exel6021	-	7573 Df(3L)Exel6094	-	7641 Df(3R)Exel6162	-	7708 Df(1)Exel6234	-
7506 Df(2L)Exel6022	-	7574 Df(3L)Exel6095	w	7642 Df(3R)Exel6163	-	7709 Df(1)Exel6235	-
7507 Df(2L)Exel6024	-	7575 Df(3L)Exel6096	-	7643 Df(3R)Exel6164	-	7710 Df(1)Exel6236	-
7508 Df(2L)Exel6025	-	7576 Df(3L)Exel6097	-	7644 Df(3R)Exel6165	-	7711 Df(1)Exel6237	-
7510 Df(2L)Exel6027	-	7577 Df(3L)Exel6098	-	7645 Df(3R)Exel6166	w	7712 Df(1)Exel6238	-
7511 Df(2L)Exel6028	w	7578 Df(3L)Exel6099	-	7646 Df(3R)Exel6167	w	7713 Df(1)Exel6239	-
7512 Df(2L)Exel6029	-	7580 Df(3L)Exel6101	-	7647 Df(3R)Exel6168	-	7714 Df(1)Exel6240	-
7513 Df(2L)Exel6030	-	7581 Df(3L)Exel6102	-	7648 Df(3R)Exel6169	-	7715 Df(1)Exel6241	-
7514 Df(2L)Exel6031	-	7582 Df(3L)Exel6103	-	7649 Df(3R)Exel6170	-	7716 Df(1)Exel6242	-
7515 Df(2L)Exel6032	-	7583 Df(3L)Exel6104	-	7650 Df(3R)Exel6171	-	7717 Df(1)Exel6244	-
7516 Df(2L)Exel6033	-	7584 Df(3L)Exel6105	-	7651 Df(3R)Exel6172	-	7718 Df(1)Exel6245	-
7517 Df(2L)Exel6034	-	7585 Df(3L)Exel6106	-	7652 Df(3R)Exel6173	-	7719 Df(1)Exel6248	-
7518 Df(2L)Exel6035	-	7586 Df(3L)Exel6107	m	7653 Df(3R)Exel6174	-	7720 Df(1)Exel6251	-
7519 Df(2L)Exel6036	-	7587 Df(3L)Exel6108	-	7654 Df(3R)Exel6175	-	7721 Df(1)Exel6253	-
7521 Df(2L)Exel6038	-	7588 Df(3L)Exel6109	-	7655 Df(3R)Exel6176	-	7722 Df(1)Exel6254	-
7522 Df(2L)Exel6039	-	7589 Df(3L)Exel6110	-	7658 Df(3R)Exel6179	-	7723 Df(1)Exel6255	-
7523 Df(2L)Exel6041	-	7591 Df(3L)Exel6112	w	7659 Df(3R)Exel6180	-	7724 Df(2L)Exel6256	-
7524 Df(2L)Exel6042	-	7593 Df(3L)Exel6114	-	7660 Df(3R)Exel6181	-	7726 Df(3R)Exel6259	-
7525 Df(2L)Exel6043	-	7594 Df(3L)Exel6115	-	7661 Df(3R)Exel6182	-	7729 Df(3L)Exel6262	-
7526 Df(2L)Exel6044	-	7595 Df(3L)Exel6116	-	7662 Df(3R)Exel6183	-	7730 Df(3R)Exel6263	-
7527 Df(2L)Exel6045	-	7596 Df(3L)Exel6117	-	7663 Df(3R)Exel6184	-	7731 Df(3R)Exel6264	-
7528 Df(2L)Exel6046	-	7597 Df(3L)Exel6118	-	7664 Df(3R)Exel6185	-	7732 Df(3R)Exel6265	-
7529 Df(2L)Exel6047	-	7598 Df(3L)Exel6119	-	7665 Df(3R)Exel6186	-	7734 Df(3R)Exel6267	-
7530 Df(2L)Exel6048	-	7599 Df(3L)Exel6120	w	7666 Df(3R)Exel6187	-	7736 Df(3R)Exel6269	-
7531 Df(2L)Exel6049	-	7600 Df(3L)Exel6121	-	7667 Df(3R)Exel6188	-	7737 Df(3R)Exel6270	-
7532 Df(2R)Exel6050	-	7601 Df(3L)Exel6122	w	7668 Df(3R)Exel6189	-	7739 Df(3R)Exel6272	-
7533 Df(2R)Exel6051	-	7602 Df(3L)Exel6123	-	7669 Df(3R)Exel6190	-	7740 Df(3R)Exel6273	-
7534 Df(2R)Exel6052	-	7604 Df(3L)Exel6125	-	7670 Df(3R)Exel6191	-	7741 Df(3R)Exel6274	-
7535 Df(2R)Exel6053	-	7605 Df(3L)Exel6126	-	7671 Df(3R)Exel6192	w	7742 Df(3R)Exel6275	-
7536 Df(2R)Exel6054	-	7606 Df(3L)Exel6127	-	7672 Df(3R)Exel6193	-	7743 Df(3R)Exel6276	-
7537 Df(2R)Exel6055	-	7607 Df(3L)Exel6128	w	7673 Df(3R)Exel6194	-	7744 Df(2L)Exel6277	-
7538 Df(2R)Exel6056	-	7608 Df(3L)Exel6129	-	7674 Df(3R)Exel6195	-	7745 Df(3L)Exel6279	-
7539 Df(2R)Exel6057	-	7609 Df(3L)Exel6130	-	7675 Df(3R)Exel6196	-	7746 Df(3R)Exel6280	-
7540 Df(2R)Exel6058	-	7610 Df(3L)Exel6131	-	7676 Df(3R)Exel6197	-	7747 Df(3R)Exel6282	-
7541 Df(2R)Exel6059	-	7611 Df(3L)Exel6132	-	7677 Df(3R)Exel6198	-	7748 Df(2R)Exel6283	-
7542 Df(2R)Exel6060	-	7612 Df(3L)Exel6133	-	7678 Df(3R)Exel6199	-	7749 Df(2R)Exel6284	-
7543 Df(2R)Exel6061	-	7613 Df(3L)Exel6134	-	7679 Df(3R)Exel6200	-	7750 Df(2R)Exel6285	-
7544 Df(2R)Exel6062	-	7614 Df(3L)Exel6135	-	7680 Df(3R)Exel6201	-	7752 Df(3R)Exel6288	-
7545 Df(2R)Exel6063	-	7615 Df(3L)Exel6136	-	7681 Df(3R)Exel6202	-	7753 Df(1)Exel6290	-
7546 Df(2R)Exel6064	-	7616 Df(3L)Exel6137	-	7682 Df(3R)Exel6203	-	7754 Df(1)Exel6291	-
7547 Df(2R)Exel6065	-	7617 Df(3L)Exel6138	-	7683 Df(3R)Exel6204	-	7759 Df(1)Exel9050	-
7548 Df(2R)Exel6066	-	7619 Df(3R)Exel6140	-	7684 Df(3R)Exel6205	-	7760 Df(1)Exel9053	-
7549 Df(2R)Exel6067	-	7620 Df(3R)Exel6141	-	7685 Df(3R)Exel6206	-	7761 Df(1)Exel7463	-
7550 Df(2R)Exel6068	-	7621 Df(3R)Exel6142	-	7686 Df(3R)Exel6208	-	7762 Df(1)Exel9051	-
7551 Df(2R)Exel6069	s	7622 Df(3R)Exel6143	w	7687 Df(3R)Exel6209	-	7763 Df(1)Exel9054	-
7552 Df(2R)Exel6070	-	7623 Df(3R)Exel6144	-	7688 Df(3R)Exel6210	-	7764 Df(1)Exel7464	-

BL# Deficiency	S	BL# Deficiency	S	BL# Deficiency	S	B L# Deficiency	S
7765 Df(1)Exel9067	-	7840 Df(2L)Exel8038	-	7923 Df(3L)Exel9058	-	7991 Df(3R)Exel9013	-
7766 Df(1)Exel7465	-	7841 Df(2L)Exel9033	-	7924 Df(3L)Exel9001	-	7992 Df(3R)Exel9014	-
7767 Df(1)Exel9068	-	7842 Df(2L)Exel9063	-	7925 Df(3L)Exel9028	-	7993 Df(3R)Exel8178	-
7768 Df(1)Exel7468	-	7843 Df(2L)Exel7071	-	7926 Df(3L)Exel7208	-	7994 Df(3R)Exel9056	-
7769 Df(1)Exel8196	-	7844 Df(2L)Exel7072	-	7927 Df(3L)Exel7210	-	7995 Df(3R)Exel9025	-
7770 Df(1)Exel9049	-	7846 Df(2L)Exel8039	-	7928 Df(3L)Exel8101	-	7997 Df(3R)Exel7378	-
7771 Df(1)Exel9052	-	7847 Df(2L)Exel8040	-	7929 Df(3L)Exel8104	-		
7772 Df(2L)Exel7002	-	7848 Df(2L)Exel7075	-	7930 Df(3L)Exel9034	-		
7774 Df(2L)Exel8003	-	7850 Df(2L)Exel7077	-	7931 Df(3R)Exel7315	-		
7775 Df(2L)Exel7005	-	7851 Df(2L)Exel7078	-	7932 Df(3R)Exel7317	-		
7776 Df(2L)Exel7006	-	7852 Df(2L)Exel7079	-	7933 Df(3L)Exel9048	-		
7777 Df(2L)Exel8004	-	7853 Df(2L)Exel7080	-	7934 Df(3L)Exel9017	-		
7778 Df(2L)Exel7007	-	7855 Df(2L)Exel7081	-	7935 Df(3L)Exel9002	-		
7779 Df(2L)Exel8005	-	7858 Df(2R)Exel7092	-	7936 Df(3L)Exel9003	-		
7780 Df(2L)Exel7008	-	7859 Df(2R)Exel7094	-	7937 Df(3L)Exel9004	-		
7782 Df(2L)Exel7010	-	7860 Df(2R)Exel7095	-	7938 Df(3L)Exel7253	-		
7783 Df(2L)Exel7011	-	7862 Df(2R)Exel7096	-	7940 Df(3L)Exel9006	-		
7784 Df(2L)Exel7014	-	7863 Df(2R)Exel8047	-	7941 Df(3L)Exel9046	-		
7785 Df(2L)Exel7015	-	7864 Df(2R)Exel7098	-	7942 Df(3L)Exel9007	-		
7786 Df(2L)Exel8008	-	7866 Df(2R)Exel8049	-	7943 Df(3L)Exel9008	-		
7787 Df(2L)Exel7016	-	7867 Df(2R)Exel9016	-	7944 Df(3L)Exel9009	-		
7789 Df(2L)Exel7018	-	7869 Df(2R)Exel7121	-	7945 Df(3L)Exel9011	-		
7790 Df(2L)Exel8010	-	7870 Df(2R)Exel7123	-	7946 Df(3L)Exel9061	-		
7792 Df(2L)Exel9062	-	7871 Df(2R)Exel8057	-	7947 Df(3L)Exel9045	-		
7793 Df(2L)Exel8012	-	7872 Df(2R)Exel7124	-	7948 Df(3R)Exel7357	-		
7794 Df(2L)Exel7022	-	7873 Df(2R)Exel7128	-	7949 Df(3L)Exel9065	-		
7795 Df(2L)Exel702	-	7875 Df(2R)Exel7130	-	7950 Df(3L)Exel9066	-		
7796 Df(2L)Exel8013	-	7876 Df(2R)Exel7131	-	7951 Df(3R)Exel9029	-		
7797 Df(2L)Exel7023	-	7877 Df(2R)Exel8059	-	7952 Df(3R)Exel7283	-		
7798 Df(2L)Exel8016	-	7879 Df(2R)Exel7135	-	7953 Df(3R)Exel7284	-		
7799 Df(2L)Exel7024	-	7880 Df(2R)Exel9015	-	7954 Df(3R)Exel8143	-		
7800 Df(2L)Exel9038	-	7881 Df(2R)Exel9026	-	7955 Df(3R)Exel9036	-		
7801 Df(2L)Exel7027	-	7882 Df(2R)Exel7137	-	7956 Df(3R)Exel7305	-		
7802 Df(2L)Exel7029	-	7883 Df(2R)Exel7138	-	7957 Df(3R)Exel7306	-		
7803 Df(2L)Exel8019	-	7884 Df(2R)Exel7139	-	7958 Df(3R)Exel8152	-		
7804 Df(2L)Exel7031	-	7885 Df(2R)Exel9060	-	7959 Df(3R)Exel7308	-		
7805 Df(2L)Exel9031	-	7886 Df(2R)Exel7142	-	7960 Df(3R)Exel7309	-		
7807 Df(2L)Exel7034	-	7887 Df(2R)Exel7145	-	7961 Df(3R)Exel8154	-		
7808 Df(2L)Exel8021	-	7888 Df(2R)Exel7144	-	7962 Df(3R)Exel9018	-		
7809 Df(2L)Exel7038	-	7890 Df(2R)Exel7149	-	7963 Df(3R)Exel8153	-		
7810 Df(2L)Exel7039	-	7891 Df(2R)Exel7150	-	7964 Df(3R)Exel9019	-		
7811 Df(2L)Exel7040	-	7893 Df(2R)Exel7153	-	7965 Df(3R)Exel7310	-		
7812 Df(2L)Exel7042	-	7894 Df(2R)Exel7157	-	7966 Df(3R)Exel7312	-		
7813 Df(2L)Exel8022	-	7895 Df(2R)Exel7158	-	7967 Df(3R)Exel8155	-		
7814 Df(2L)Exel9064	-	7896 Df(2R)Exel7162	-	7968 Df(3R)Exel7313	-		
7815 Df(2L)Exel9040	-	7897 Df(2R)Exel7163	-	7969 Df(3R)Exel7314	-		
7816 Df(2L)Exel7043	-	7898 Df(2R)Exel7164	-	7970 Df(3R)Exel7316	-		
7817 Df(2L)Exel8024	-	7900 Df(2R)Exel7169	-	7972 Df(3R)Exel7318	-		
7818 Df(2L)Exel9032	-	7901 Df(2R)Exel7170	-	7973 Df(3R)Exel8157	-		
7819 Df(2L)Exel7046	-	7902 Df(2R)Exel7171	-	7974 Df(3R)Exel8158	-		
7820 Df(2L)Exel8026	-	7903 Df(2R)Exel7173	-	7975 Df(3R)Exel7320	-		
7821 Df(2L)Exel7049	-	7904 Df(2R)Exel7174	-	7976 Df(3R)Exel8159	-		
7822 Df(2L)Exel8028	-	7905 Df(2R)Exel7176	-	7977 Df(3R)Exel7321	-		
7823 Df(2L)Exel7055	-	7906 Df(2R)Exel7177	-	7978 Df(3R)Exel8160	-		
7826 Df(2L)Exel7059	-	7909 Df(2R)Exel7180	-	7980 Df(3R)Exel7326	-		
7828 Df(2L)Exel8033	-	7910 Df(2R)Exel7182	-	7981 Df(3R)Exel8162	-		
7830 Df(2L)Exel8034	-	7912 Df(2R)Exel7184	-	7982 Df(3R)Exel7327	-		
7831 Df(2L)Exel7063	-	7913 Df(2R)Exel9043	-	7983 Df(3R)Exel7328	-		
7833 Df(2L)Exel7066	-	7914 Df(2R)Exel7185	-	7984 Df(3R)Exel7329	-		
7834 Df(2L)Exel7067	-	7916 Df(2L)Exel8056	-	7985 Df(3R)Exel7330	-		
7835 Df(2L)Exel8036	-	7918 Df(3R)Exel8194	-	7986 Df(3R)Exel9055	-		
7836 Df(2L)Exel9044	-	7919 Df(3R)Exel7379	-	7987 Df(3R)Exel8163	-		
7837 Df(2L)Exel7069	-	7920 Df(3L)Exel9057	-	7988 Df(3R)Exel8165	-		
7838 Df(2L)Exel7068	-	7921 Df(3L)Exel9000	-	7989 Df(3R)Exel9030	-		
7839 Df(2L)Exel7070	-	7922 Df(3L)Exel8098	-	7990 Df(3R)Exel9012	-		

BL# = Bloomington stock #
S = Suppression?

- = no suppression
w = weak
m = medium
s = strong

When loss of function alleles available, these deficiencies were confirmed to be correct.
(by the Bloomington Drosophila Stock Center)

6.4 Tested DrosDel Deficiencies

Deficiency	S	Deficiency	S	Deficiency	S	Deficiency	S
Df(1)ED404	-	Df(2L)ED49	-	Df(3L)ED4238	-	Df(3R)ED5221	-
Df(1)ED418	-	Df(2L)ED499	-	Df(3L)ED4256	-	Df(3R)ED5223	-
Df(1)ED429	-	Df(2L)ED508	-	Df(3L)ED4284	-	Df(3R)ED5230	-
Df(1)ED6443	-	Df(2L)ED5878	-	Df(3L)ED4287	-	Df(3R)ED5296	m
Df(1)ED6574	-	Df(2L)ED606	-	Df(3L)ED4288	-	Df(3R)ED5300	-
Df(1)ED6630	-	Df(2L)ED62	-	Df(3L)ED4293	-	Df(3R)ED5516	-
Df(1)ED6712	-	Df(2L)ED623	-	Df(3L)ED4342	-	Df(3R)ED5558	-
Df(1)ED6720	-	Df(2L)ED647	-	Df(3L)ED4408	w	Df(3R)ED5608	-
Df(1)ED6829	-	Df(2L)ED680	-	Df(3L)ED4415	-	Df(3R)ED5622	-
Df(1)ED6906	-	Df(2L)ED695	-	Df(3L)ED4416	-	Df(3R)ED5705	-
Df(1)ED7005	-	Df(2L)ED700	-	Df(3L)ED4421	-	Df(3R)ED5785	-
Df(1)ED7153	-	Df(2L)ED746	-	Df(3L)ED4470	-	Df(3R)ED5911	-
Df(1)ED7217	-	Df(2L)ED784	-	Df(3L)ED4483	-	Df(3R)ED5938	-
Df(1)ED7229	-	Df(2L)ED80	-	Df(3L)ED4486	-	Df(3R)ED5942	-
Df(1)ED7374	w	Df(2L)ED800	-	Df(3L)ED4502	-	Df(3R)ED6076	-
Df(1)ED7413	-	Df(2L)ED87	-	Df(3L)ED4543	-	Df(3R)ED6085	-
Df(2L)ED1050	-	Df(2L)ED929	-	Df(3L)ED4674	-	Df(3R)ED6103	-
Df(2L)ED1109	-	Df(2R)ED1	-	Df(3L)ED4685	-	Df(3R)ED6232	-
Df(2L)ED1200	-	Df(2R)ED1552	-	Df(3L)ED4782	-	Df(3R)ED6237	-
Df(2L)ED1231	-	Df(2R)ED1612	-	Df(3L)ED4786	-	Df(3R)ED6242	-
Df(2L)ED1303	-	Df(2R)ED3923	-	Df(3L)ED4789	-	Df(3R)ED6310	-
Df(2L)ED1305	-	Df(3L)ED201	-	Df(3L)ED4799	-	Df(3R)ED7665	-
Df(2L)ED134	-	Df(3L)ED202	-	Df(3L)ED4978	-		
Df(2L)ED136	-	Df(3L)ED207	-	Df(3L)ED5017	-	S = Suppression?	
Df(2L)ED1466	-	Df(3L)ED208	-	Df(3R)ED2	-		
Df(2L)ED19	-	Df(3L)ED210	-	Df(3R)ED5020	-	- = no suppression	
Df(2L)ED270	-	Df(3L)ED211	-	Df(3R)ED5021	-	w = weak	
Df(2L)ED273	-	Df(3L)ED212	-	Df(3R)ED5046	-	m = medium	
Df(2L)ED279	-	Df(3L)ED215	-	Df(3R)ED5066	-		
Df(2L)ED284	-	Df(3L)ED217	-	Df(3R)ED5071	-		
Df(2L)ED285	-	Df(3L)ED218	-	Df(3R)ED5092	-		
Df(2L)ED292	-	Df(3L)ED219	-	Df(3R)ED5095	-		
Df(2L)ED299	-	Df(3L)ED220	-	Df(3R)ED5100	-		
Df(2L)ED343	-	Df(3L)ED223	-	Df(3R)ED5138	-		
Df(2L)ED353	-	Df(3L)ED224	m	Df(3R)ED5142	-		
Df(2L)ED354	-	Df(3L)ED225	m	Df(3R)ED5147	-		
Df(2L)ED369	-	Df(3L)ED228	-	Df(3R)ED5156	-		
Df(2L)ED384	-	Df(3L)ED230	-	Df(3R)ED5177	-		
Df(2L)ED475	-	Df(3L)ED231	-	Df(3R)ED5196	-		
Df(2L)ED479	-	Df(3L)ED4079	-	Df(3R)ED5220	-		

6.5 Genetic Evidence For a Role of *djun* and *dfos* in Eiger Signaling

Since loss of *djun* and *dfos* does not dominantly suppress the Eiger-induced small eye phenotype, we tested their recessive potential to do so. For this purpose we used the *eyflp* system to generate flies that have *djun* or *dfos* homozygous mutant heads but are otherwise heterozygous. As a control we also generated such animals with a *bsk* mutant allele (bsk^1), although *bsk* already dominantly suppresses the small eye. Since *djun* and *bsk* are located on the second chromosome, we needed to use a combination of *GMR-Gal4* and *UAS-egr* on the third chromosome (Figure 12A, and B), which is weaker and more variable than the combination of insertions on the second chromosome (Figure 12G). Mosaic flies with homozygous eyes mutant for *bsk* or *djun* display a suppression of this weak small eye phenotype (Figure 12C, and E). The eye size in control *bsk* or *djun* eyeflp clones is not affected (Figure 12D, and F). Co-expression of a dominant negative form of dFos ($dFos^{bZIP}$) also suppresses the small eye phenotype (Figure 12H). Since the eyes of flies that express *GMR-egr* and are homozygous mutant for *dfos* exhibited a very distorted eye (not shown) this experiment was not informative. Moreover, control *dfos eyflp* clones could not be obtained. Second hits on the *dfos* mutant chromosome are a possible explanation for this observation.

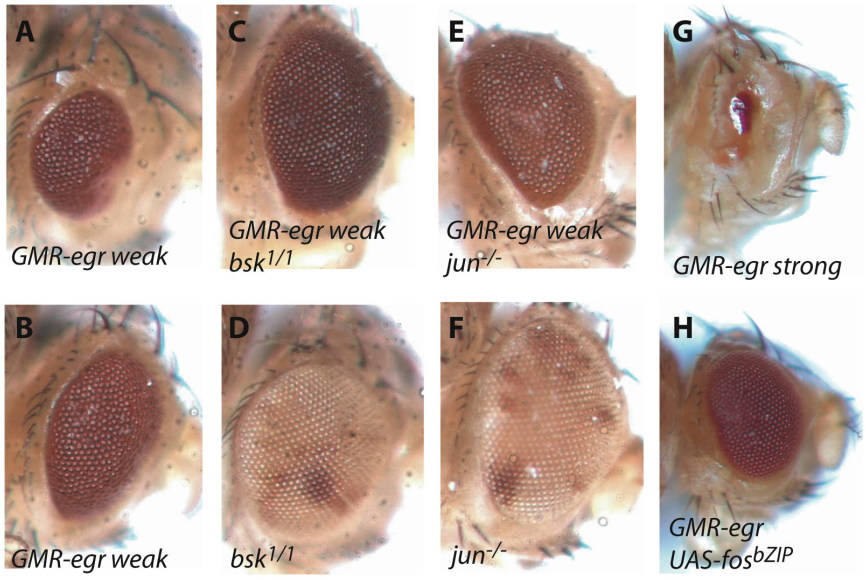

Figure 12 (A)+(B) *GMR-Gal4(III) UAS-egr(III)/+*. The combination of *GMR-Gal4* and *UAS-egr* on the third chromosome is weaker and quite variable compared to the one on the second chromosome. (C) *eyflp; FRT* bsk^1*/FRT cl; GMR-egr(III)*. Mosaic flies with eyes homozygous mutant for *bsk* display a good suppression of the Eiger-induced small eye phenotype. (D) *eyflp; FRT* bsk^1*/FRT cl; +. bsk* control *eyflp* clone (E) *eyflp; FRT* jun^-*/FRT cl; GMR-egr(III)*. Mosaic flies with eyes homozygous mutant for d*jun* also display a good suppression of the Eiger-induced small eye phenotype. (F) *eyflp; FRT* $djun^-$*/FRT cl; +. jun* control *eyflp* clones (G) Strong *GMR-Gal4 UAS-egr* combination on the second chromosome. (H) *GMR-egr/+;* $UAS\text{-}fos^{bZIP}$*/+*. Co-expression of a dominant negative version of dFos suppresses the small eye phenotype.

6.6 Characterization of an *egr-lacZ* Reporter *in vivo*

In order to generate an *egr-lacZ* reporter line, transgenic flies were generated carrying a 10kb fragment of the *egr* locus where the *lacZ* coding region has been inserted at the position of the ATG of the *egr* gene (*714-lacZ*, D. Hengartner, unpublished). Imaginal discs from these lines displayed a *lacZ* expression pattern identical to the *egr* RNA *in situ* expression pattern, indicating that all the regulatory sequences required for *egr* imaginal disc expression under normal conditions are included in this 10kb fragment (Figure 13). To further narrow down the regulatory region of *egr*, sub-fragments (pDA679, pDA690, pDA676, pDA675, D. Hengartner, unpublished) of this 10kb fragment were cloned into a *lacZ*-containing transformation vector (pX27). Of the four fragments tested, only one, pDA679, produced a strong very specific *egr*-like expression pattern in imaginal discs (Figure 13). These *egr* reporter lines may be very useful when studying the transcriptional regulation of *egr*. Since no eye- or wing-phenotype could be observed in *egr* mutants, the physiological importance of the observed expression pattern remains to be elucidated.

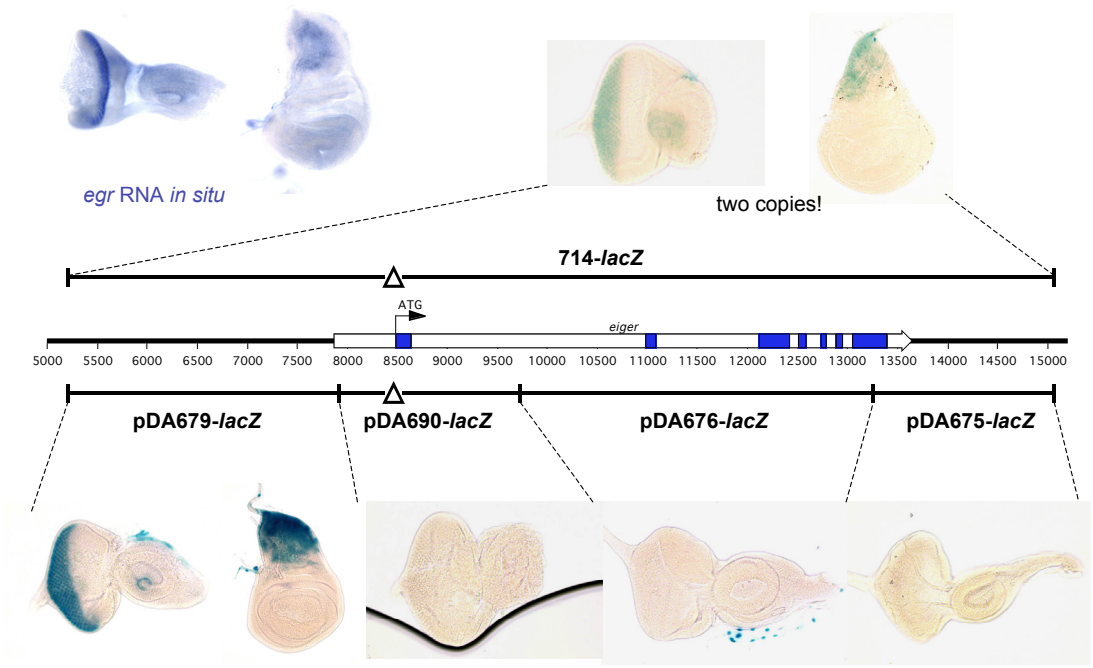

Figure 13 Schematic overview over the *egr* locus and the fragments used to generate an *egr-lacZ* reporter line.

6.7 Eiger Overexpression in the Embryo

In this study we used the potential of Eiger to induce JNK-mediated apoptosis in *Drosophila* imaginal discs. To test whether Eiger also induces apoptosis at embryonic stages, *UAS-egr* was expressed under the control of *prd-Gal4*. Surprisingly such flies survived to adulthood without any morphological defects (besides a very mild phenotype at the tip of the wing due to the leakiness of the Gal4 driver) indicating that Eiger is not able to induce apoptosis in the embryo. To test whether this is due to the failure of Eiger to induce JNK in embryos or due to a different output of JNK signaling, JNK activation was measured by *puc-lacZ* staining. A subset of the progeny of the cross depicted in Figure 14 are embryos expressing *UAS-egr* under the control of *prd-Gal4* that also carry the *puc-lacZ* reporter. All embryos that displayed a lacZ staining reflected only the endogenous *puc* expression that is required in the process of dorsal closure. A *prd*-like expression pattern in stripes was never observed. Since adult flies expressing *UAS-egr* under the control of *prd-Gal4* were obtained, this is not due to the lack of such embryos, but indeed indicated that Eiger fails to induce JNK activation in the embryo. This is consistent with the observation that also in embryo-derived S2- or Kc-cells Eiger does not induce JNK phosphorylation (Narasimamurthy, 2006), which may be due to the lack of expression of critical pathway components in the embryonic stage.

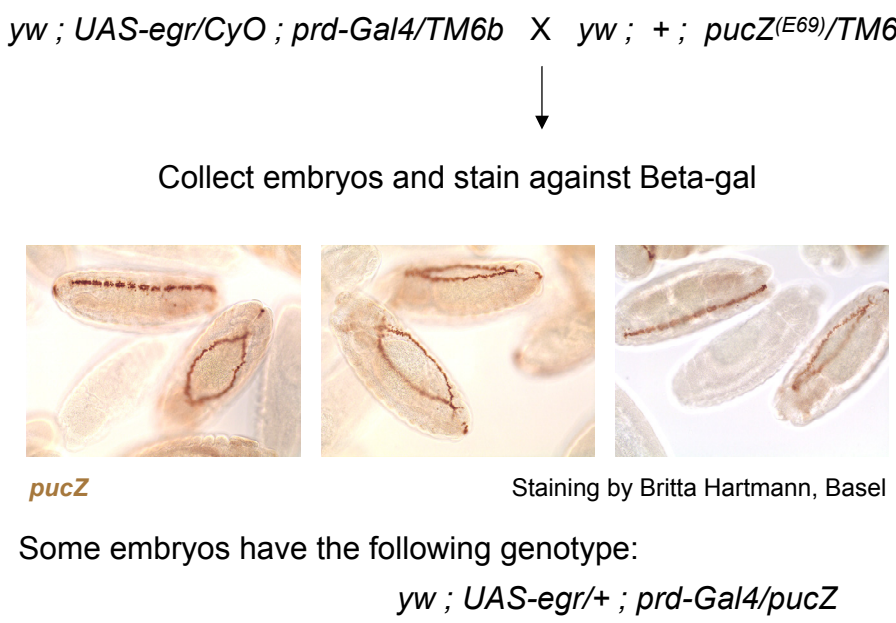

Figure 14 Expression of *UAS-egr* under the control of *prd-Gal4* does not induce ectopic JNK activation in the embryo.

6.8 Attempts to Generate a *hid-lacZ* Reporter

Since the pro-apoptotic gene *hid* was shown to be transcriptionally upregulated in eye discs expressing *UAS-egr* under the control of *GMR-Gal4* (Moreno et al., 2002b), we were interested in identifying the regulatory sequences that are responsive to the Eiger signal. For this purpose two 5kb fragments covering 10kb upstream of the *hid* translational start and two 5kb fragments covering the first large intron were cloned into a *lacZ*-containing transformation vector (pX27). None of these reporter fragments displayed a lacZ staining either in a wildtype or in a *GMR-egr* background (not shown). Interestingly other attempts to generate a similar reporter line also failed (Moon et al., 2005), suggesting that the *hid* regulatory region may be very complex, which is also supported by the fact that the *hid* 5'UTR (~10kb) and first exon (~10kb) are very large. Another aspect is, that locus 75C, where *hid* is located, contains three further pro-apoptotic genes (*rpr, grim, skl*) of which at least one is also involved in Eiger-induced apoptosis (see Chapter 4.2). This raises the possibility that not only *hid* but the whole locus is under complex transcriptional regulation.

6.9 *eiger* Mutants Under Different Stress Conditions

egr mutants are homozygous viable and do not display any obvious phenotype. As discussed in Chapter 5.4 this could be due to the fact that the phenotype is very subtle and therefore hard to detect or that a phenotype will only be observed under certain conditions. So far only one such condition was reported to have an effect. *egr* mutants have a delay in lethality caused by Salmonella infection (Brandt et al., 2004), which is not dependent on the canonical innate immunity pathways Toll and IMD. Whether this is due to the lack of Eiger-mediated JNK induction remains to be confirmed by testing flies homozygous mutant for *dTAK1, dTAB2* or *Mkk4*. The Toll- and IMD-dependent innate immune response to gram-positive/negative bacteria or fungi is not affected in *egr* mutants (B. Lemaitre, personal communication). Since Eiger induces JNK and subsequent apoptosis, we also tested whether *egr* mutants are more susceptible to oxidative stress, which is dependent on JNK signaling (Wang et al., 2003), or whether they have a reduced capability to mediate X-ray-induced apoptosis. No difference in the response to Paraquat (oxidative stress) could be observed in comparison to wildtype flies (not shown). Similarly in response to X-ray treatment (5000 rad) cleavage of caspase-3 is not affected (Figure 15). Moreover *egr* is not transcriptionally upregulated in response to X-ray irradiation (Figure 15).

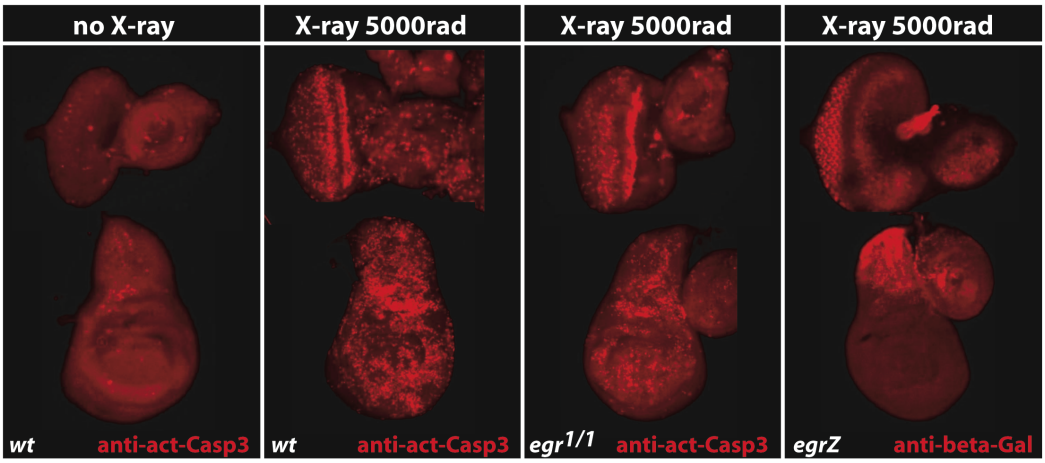

Figure 15 X-ray-induced Caspase-3 cleavage is not perturbed in *egr* mutants and *egr* is not transcriptionally upregulated in response to X-ray.

6.10 Further Genes Tested for Suppression of the Small Eye

Gene:	**Genotype:**	**Suppression:**	**Allele Source:**
IMD:	*IMD*$^{-/-}$ *; GMR-egr*	no suppression	B. Lemaitre
dFADD:	*GMR-egr; dFADD*$^{-/-}$	no suppression	Naitza et al. (2002)
Toll (Tl):	*GMR-egr; Tl*$^{-/-}$	no suppression	B. Lemaitre
pelle (pll):	*GMR-egr; pll*$^{-/-}$	no suppression	B. Lemaitre
Relish (Rel):	*GMR-egr; Rel*$^{-/-}$	no suppression	B. Lemaitre
p38a:	*GMR-egr; p38a*$^{-/-}$	no suppression	Craig et al. (2004)
dMEKK1:	*GMR-egr; dMEKK1*$^{-/-}$	no suppression	Inoue et al. (2001)
dMyD88:	*GMR-egr; dMyD88*$^{-/-}$	no suppression	Charatsi et al. (2003)
MAPk-Ak2:	*MAPk-Ak2*$^{-/Y}$*; GMR-egr*	no suppression	G. Seisenbacher

7 Materials

7.1 Fly Stocks

Balanced EMS-induced suppressor mutations:

Stocks carrying an allele on the second chromosome might have *Mkrs, TM6b* or y^+ floating on the third. Some still carry the *GMR-Gal4*(w^+) insertion on the second chromosome.
Stocks carrying an allele on the third chromosome are clean for the second chromosome.

(I): genotype associated with chromosome I
(II): genotype associated with chromosome II
(III): genotype associated with chromosome III
BL: Bloomington Stock Number

Gal4 driver:

GMR-Gal4 (II) strong	bK376
GMR-Gal4 (II) weak	K627
GMR-Gal4 (III) weak	K628
prd-Gal4 (III)	bK387
wing-Gal4 (III) (C765, line 10)	K369

UAS transgenes:

UAS-egr (II) strong	Moreno et al. (2002b)
UAS-egr (III) weak	Moreno et al. (2002b)
UAS-hep^{CA} (II)	Adachi-Yamada et al. (1999)
EP-hid (III)	-
UAS-Egfr^{DN} (II)	MF806 (Freeman Lab)
UAS-lilli (II)	Wittwer et al. (2001)
UAS-djun (II)	Hafen Lab
UAS-dfos (II)	BL-7213
UAS-dfos^{bZIP} (III)	Marek Mlodzik
UAS-Inr (II)	Brogiolo et al. (2001)
UAS-dgadd (II)+(III)	this thesis
UAS-dTAB2 (II)+(III)	this thesis
tub-dTAB2 (III)	this thesis
UAS-Mkk4 (II)+(III)	this thesis
tub-Mkk4 (II)+(III)	this thesis
UAS-Mkk4^{Asp} (II)+(III)	this thesis
UAS-Mkk4^{Mut} (II)+(III)	this thesis

Combinations of Gal4 and UAS transgenes:

GMR-Gal4 (strong) *UAS-egr/CyO*

GMR-Gal4 (strong) *UAS-egr/CyO; Mkrs/TM6b*

GMR-Gal4 (weak) *UAS-egr/TM6b*

Sp/CyO; GMR-Gal4 (weak) *UAS-egr/TM6b*

UAS-egr; prd-Gal4

GMR-Gal4 (weak) *UAS-hep^{CA}/CyO*

GMR-Gal4 (weak) *UAS-hep^{CA}/CyO: Mkrs/TM6b*

GMR-Gal4 (weak) *EP-hid/TM6b*

GMR-Gal4 (strong) *UAS-Egfr^{DN}*/CyO

GMR-Gal4 (strong) *UAS-Inr*/CyO

Others:

UAS-egr; y^{+}	this thesis
Df(2R)BSC26 (II)	BL-6866
Df(2L)C144 (II)	BL-90
bsk^{1} (II)	Hafen Lab
hep^{1} (I)	Noselli Lab
msn^{102} (III)	Hafen Lab
lilli^{4U5,15D1} (II)	Wittwer et al. (2001)
egr^{1,3} (II)	Igaki et al. (2002)
dTAK1^{1,2,3,4} (I)	Vidal et al. (2001)
dTRAF1^{ex1} (II)	Cha et al. (2003)
dTRAF2^{ex1} (I)	Cha et al. (2003)
EP2L (II)	Zipperlen et al. (2005)
FRT40 (II)	Zipperlen et al. (2005)
RFP4,5,15 (III)	J. Bischof
f07920 (III)	Exelixis
d03195 (III)	Exelixis
P[dTAK1] (III) genomic rescue transgene	Vidal et al. (2001)
P[egr] (II)+(III) genomic rescue transgene (713)	D. Hengartner
679-lacZ (II)+(III) *egrZ*	this thesis
714-lacZ (II)+(III) *egrZ*	D. Hengartner
2x714-lacZ (II) *egrZ*	this thesis

7.2 Constructs

pUAST-dTAB2	PCR (PG164+PG165) on EST LD40663 cloned into pMZ55 Asp718 X HindIII cut out with Asp718(blunt) X NotI cloned into pUAST ExoRI(blunt) X NotI
pUAST-HA-dTAB2	PCR (PG163+PG164) on EST LD40663 cloned into pMZ55 NheI X HindIII cut out with Asp718(blunt) X NotI cloned into pUAST ExoRI(blunt) X NotI
tub-dTAB2	PCR (PG164+PG165) on EST LD40663 cloned into pMZ55 Asp718 X HindIII cut out with Asp718 X NotI cloned into pOP118 Asp718 X NotI
tub-HA-dTAB2	PCR (PG163+PG164) on EST LD40663 cloned into pMZ55 NheI X HindIII cut out with Asp718 X NotI cloned into pOP118 Asp718 X NotI
pUAST-Mkk4	PCR (PG171+PG172) on EST RE70055 cloned into pMZ55 Asp718 X HindIII (=pMZ55-Mkk4) cut out with Asp718(blunt) X NotI cloned into pUAST ExoRI(blunt) X NotI
pUAST-HA-Mkk4	PCR (PG170+PG171) on EST RE70055 cloned into pMZ55 NheI X HindIII (=pMZ55-HA-Mkk4) cut out with Asp718(blunt) X NotI cloned into pUAST ExoRI(blunt) X NotI
tub-Mkk4	PCR (PG171+PG172) on EST RE70055 cloned into pMZ55 Asp718 X HindIII cut out with Asp718 X NotI cloned into pOP118 Asp718 X NotI
tub-HA-Mkk4	PCR (PG170+PG171) on EST RE70055 cloned into pMZ55 NheI X HindIII cut out with Asp718 X NotI cloned into pOP118 Asp718 X NotI

pUAST-Mkk4^{Asp}	annealed primer Mkk4^{Asp}_fw + Mkk4^{Asp}_rev cloned into pMZ55-Mkk4 PstI X SalI cut out with Asp718(blunt) X NotI cloned into pUAST EcoRI(blunt) X NotI
pUAST-HA-Mkk4^{Asp}	annealed primer Mkk4^{Asp}_fw + Mkk4^{Asp}_rev cloned into pMZ55-HA-Mkk4 PstI X SalI cut out with Asp718(blunt) X NotI cloned into pUAST EcoRI(blunt) X NotI
pUAST-Mkk4^{Mut}	annealed primer Mkk4^{Mut}_fw + Mkk4^{Mut}_rev cloned into pMZ55-Mkk4 PstI X SalI cut out with Asp718(blunt) X NotI cloned into pUAST EcoRI(blunt) X NotI
pUAST-HA-Mkk4^{Mut}	annealed primer Mkk4^{Mut}_fw + Mkk4^{Mut}_rev cloned into pMZ55-HA-Mkk4 PstI X SalI cut out with Asp718(blunt) X NotI cloned into pUAST EcoRI(blunt) X NotI
pUAST-dgadd (CG11086)	PCR (PG101+102) on yw DNA cloned into pKB342 Asp718 X EcoRI cut out with Asp718 X XbaI cloned into pUAST Asp718 X XbaI
pDA675-pX27	cloned into pX27(XbaI→NotI) BamHI X NotI
pDA676-pX27	pDA676 KspI(blunt) X BamHI cloned into pX27 EcoRI(blunt) X BamHI
pDA679-pX27	cloned into pX27(XbaI→Asp718) BamHi X Asp718
pDA690-pX27	cloned into pX27 BamHI X XhoI
hidF1-pX27	PCR (PG111+PG112) on yw DNA cloned into pX27 XbaI X EcoRI
hidF2-pX27	PCR (PG105+PG106) on yw DNA cloned into pX27 EcoRI

hidF3-pX27	PCR (PG107+PG108) on yw DNA cloned into pX27 XbaI X EcoRI
hidF4-pX27	PCR (PG109+PG110) on yw DNA cloned into pX27 XbaI

7.3 Primers

***bsk* sequencing:**

bsk1	CCACCCATTCAGAATGGAGC
bsk2	CGGCCTTTACAACTATATTGG
bsk3	CGACAGAATGTCCTATTTGC
bsk4	CAATACACGCCGTATTGTTGG

***dTAK1* sequencing:**

dTAK1-f1	CAAGGAATCGGTGGCATAC
dTAK1-r1	CTGGTTATGCGGAATAGC
dTAK1-f2	GGTTATAACGTAGGGCTC
dTAK1-r2	CGAGCAGTTCAAGGATC
dTAK1-f3	GCGTTCTTTTACTCTGCC
dTAK1-r3	CCATTGTGTCTCTGAACG
dTAK1-f4	GTTCGAGGCTGTCGAAGTTC
dTAK1-r4	CGTACAGATACAGACTACG

***dTAB2* sequencing:**

PG153	GAACAACAGTCAGGAGCTAGC
PG154	CGCAAGGCAATCATTTGTTGC
PG155	CCATCTTCTGCTAAAACTACG
PG156	GCGTCAAAACTAACGCTGG
PG156.2	GTTGCGTACAAGGTCCTTGG
PG157	GCTTCATCCTATCCCAGTGC
PG157.2	CCAGATGTTGAGGAAGGAGC
PG158	GGGCAATGGTATAATCCCG
G867_mut	GAGACACGAGGAATCGTTGG

***dTAB2* cloning and construct sequencing:**

PG163	AAAAGCTAGCATGGCGGCTACACCACCAATGC
PG164	AAAAAAGCTTTTATGTATGCAGAGCGTACGGC
PG165	AAAGGTACCGCCACCATGGCGGCTACACCACCAATGC
sequencing:	
PG166	CCAGATGTTGAGGAAGGAGC
PG167	GCAGCTTCTCCAACGATTCC
PG168	GTTAGTTTTGACGCACGTCG
PG169	CACTATCGAGCGTCAGAAGC
seq_HA_GAP	CCTGTTTCATGAGATGAAGC

plus standard primers: tub prom, tub-1, M13

***Mkk4* sequencing:**

Mkk4_seq1	CGGACAAAACATCCTTAGG
Mkk4_seq2	GCATGCTGTTGTGTTCTTC
Mkk4_seq3	CAAGCTACAGTTTGGTGAGG
Mkk4_seq4	CAACATTCTGTTGCACCGTCG
Mkk4_seq5	CAAAGGAGCATCAAGTCG
Mkk4_seq6	GGTCATCTTGTTGACTGCCC
Mkk4_seq7	GAGCGCATTGATCCAGAACG
Mkk4_seq8	GGAAATTACCAGTCGCCACC
Mkk4_seq9	CTGCTTTCCTTCTGCTTAG

***Mkk4* cloning and construct sequencing:**

PG170	AAAAGCTAGCATGGCCGAACGACCGAAAAATTTG
PG171	AAAAAAGCTTTTAACTCTCCGCCTGCTGATTGGC
PG172	AAAGGTACCGCCACCATGGCCGAACGACCGAAAAATTTG
sequencing:	Mkk4_seq3,6,7,8 and standard primers: tub prom, tub-1, M13

***Mkk4*^{Asp} and *Mkk4*^{Mut}:**

Mkk4^{Asp}_fw	TCGAcgatattgccaaggataaggatgcgggcTGCA
Mkk4^{Asp}_rev	gcccgcatccttatccttggcaatatcg
Mkk4^{Mut}_fw	TCGAcgctattgccaaggtcaaggatgcgggcTGCA
Mkk4^{Mut}_rev	gcccgcatccttgaccttggcaatagcg

***lilli* sequencing:** (oFW primers kindly provided by F. Wittwer)

lilli_seqA	CCTACGTATGTGAAGCAGGC
lilli_seqB	GCAGCAATAGTTCCGAGTCC
lilli_seqC	CATTCTGGATTCAAGCGACG
lilli_seqD	GTCAAGCTGAACAAGACTGG
lilli_seqE	TGTTGCCGTTGGCAATGTCC
oFW22	CCTCGACCTTAACCCTTTGG
oFW23	AGGCAAGCCAAATGCCTCTG
oFW24	GTGACTCCGGAAGCTGCTTTG
oFW25	AAATTCAAGCCCGGCAATGTC
oFW27	ATAGTTGCTAATTTGGCCTGAC
oFW28	CGGCCATTGTTAAGAACGAG
oFW32	AGTTGCTGTTGCTGGAACTG
oFW36	CCAGCCACAAGGCTTATATAC
oFW37	CCATTCTCAGGTAGATCATTAC
oFW39	CAGCGACATAGCAAATATCTT
oFW40	TAAGCAAGCTTTCGCGAGAG
oFW41	TCATGGGACTAGGTGGTAATG
oFW47	GAAATAATGGCTTAAACCAGTAC

***hid, rpr, grim, skl* sequencing:**

hid_ex1_fw	gcaaagtgccaacaaagtgc
hid_ex1_rev	cgtaagcagttattctgtgtgg
hid_ex2_fw	gggtgatctttattacggg
hid_ex2_rev	caggcagtgtatcttttggc
hid_ex3_fw	ccgaatccgaatccgaaccg
hid_ex3_rev	ggcaggcaatgaatgctaac
hid_ex4_fw	tggttcggtcacgtgtgtgg
hid_ex4_rev	tcgttgcacttatgtacgg
rpr_fw	gaacaaagtgaacgaactcg
rpr_rev	ggttttgggttggctcatgc
grim_fw	ccaagtgctcgatacgaaac
grim_rev	tcgttcctcctcatgtgtcc
skl_fw	gaaataccttgccacacatcg
skl_rev	gtctcgctcattctctctcg

***hid* reporter:**

PG105 (hidF2) GCgaattcGGTATGTAGTATGTTCCCC

PG106 (hidF2) GCgaattcCCTTCCTGCACTTTGTTGGC

PG107 (hidF3) GCtctagaCCACACAGAATAACTGCTTACG

PG108 (hidF3) GCgaattcGGAATTGCATTTGCGACTGG

PG109 (hidF4) GCtctagaGCTGTTGAGACGTGCAGTTTGC

PG110 (hidF4) GCtctagaGCAGAAGTACGTGTATCTTACC

PG111 (hidF1) GCtctagaGGATCTGGATCTCAATCTG

PG112 (hidF1) GCgaattcCTCGAACTCGAACTCGAACG

***dgadd (CG11086)* cloning:**

PG101 gcggtaccaccatggtcgtcgaggagaactgc

PG102 gcgaattctacacagccggcagctggacaatgg

8 Methods

8.1 Molecular Cloning

Standard molecular biology techniques are described in Sambrook J. and Russell D.W., Molecular Cloning - A Laboratory Manual. (2001) Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

8.2 EMS Treatment

Drosophila males were starved for 8 hours before mutagenesis. These males were then kept for 24 hours in a bottle containing a filter paper soaked with 0.4% EMS in sugar solution (1g/100ml). After a recovery phase of another 24 hours on normal food, the mutagenized males were mated at 25°C with virgins.

8.3 Paraquat Treatment

For oxidative-stress challenge, flies were starved in empty vials for 6 h and then transferred to vials containing a gel of phosphate-buffered saline (PBS), 10% sucrose, 0.8% low-melt agarose and 20 mM paraquat. Paraquat was added to the solution after cooling down to 40°C. A control population of flies was placed in vials containing the PBS-sucrose gel without paraquat.

8.4 lacZ staining

To detect ß-galactosidase activity, third instar larval discs were fixed in 1% glutaraldehyd and subjected to the following standard X-gal color reaction:

1ml Buffer B (1mM $MgCl_2$, 10mM NaPi (Natriumphosphate-buffer pH 7), 150mM NaCl)

10µl Potassium Ferricyanide $K_3[Fe(CN)_6]$ (333mM)

10µl Potassium Ferrocyanide $K_4[Fe(CN)_6]3H_2O$ (333mM)

5µl 10% Triton-X-100

10µl 8% X-Gal

8.5 Immunohistochemistry

Imaginal discs dissected from late third instar larvae were fixed and stained with appropriate antibodies. The following antibodies were used: 1° rabbit polyclonal anti-β-galactosidase (1/2000, Cappel), 1° rabbit polyclonal anti-cleaved-caspase-3 (1/400, Cell Signaling), 2° Alexa Fluor 594 goat anti-rabbit (1/200, Molecular Probes).

For biochemistry and cell culture methods see Narasimamurthy (2006) and Geuking et al. (2005).

9 Literature

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2003 Third Symposium of the *European Community Research Training Network* “Modulation of Signaling Casacdes for the Treatment of cancer, Diabetes and Inflammation” in Athen, Griechenland, November 29, 2003.
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